Analytical studies of the tetracycline group of antibiotics

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ANALYTICAL STUDIES OF THE TETRACYCLINE GROUP OF ANTIBIOTICS

submitted by

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This research has been carried out in the school of Pharmacy and Pharmacology of the University of Bath, under the combined supervision of Dr. A.F. Casy and Dr. T.M. Jefferies.

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IN THE NAME OF ALLAH
THE MERCIFUL THE COMPASSIONATE

اَلْحَمْدُ لِلّٰهِ رَبِّ الْعٰلَمِيْنَ

وَالصَّلٰوةُ وَالسَّلَامُ عَلٰى سَيِّدِنَا مُحَمَّدٍ خَاتَمِ النَّبِيّٖنَ

Our experiments are not carried out to decide if we are right, but to gain new knowledge. It is for knowledge sake that we plough and sow. It is not inglorious at all to have erred in theories and hypotheses. Our hypotheses are intended for the present rather than the future. They are indispensable for us in the explanation of the secured facts, to enliven and to mobilise them and above all, to blaze a trail into unknown regions towards new discoveries.

R. Willstatter (1957)

Dedicated to my parents for their

continuous support and encouragement.

Mere words of gratitude are but a poor expression of my appreciation to Dr. A.F. Casy and Dr. T.M. Jefferies for all the help, guidance, encouragement and useful discussions throughout this thesis.

Sincere thanks are due to Mr. R. Hartell for his help in operating the ^{13}C NMR spectrometer, to Mr. D. Wood for carrying out the ^{1}H NMR work, to Ms. D. Belk for her assistance in the HPLC work and to all those members of the staff within the school who have beeen involved in any discussion concerning the work. I am also indebted to Miss S. Hancock and Mr. R. Sadler; without their help this thesis would never have finished.

I also wish to record my sincere appreciation to Students Supervisory Services, especially Mr. J. Taylor and the Government of Dubai for financial and moral support throughout my research. I wish to extend special thanks to Miss A.M. Ambrose for all her help and encouragement.

SUMMARY

NUCLEAR MAGNETIC RESONANCE (^{1}H AND ^{13}C NMR)

^{1}H NMR

The ^{1}H NMR spectra of tetracyclines has been studied. The protons were divided in different groups and assigned with help from model data. Most of the assignments were carried out using 60 and 100 MHz spectra, although reference was often made to high resolution spectra (220 and 400 MHz).

High resolution spectra (above 200 MHz) were employed to study the stereochemistry of tetracyclines in solution. A conformation was proposed after studying the coupling constants and the dihedral angles, in which the rings B-D were in one mean plane with the ring A "bent" at right angles to the rest of the molecule. Most of the compounds shared the same conformation although there may be exceptions e.g. 4-epi CTC HCl and OTC base.

^{13}C-NMR

The ^{13}C-NMR assignments of tetracyclines have been confirmed by relaxation times (T_1) which are influenced by the number of protons in the immediate vicinity of the carbon in question. Experiments were carried out, using different irradation modes, to determine whether the main mechanism of relaxation of the tetracyclines is via dipole-dipole interaction. The relaxation times (T_1) of TC, OTC and doxycycline were measured using the inversion recovery method.

^{13}C-NMR assignments were carried out using spectra run at 22.5 and 67.8 MHz. Assignments were confirmed with ^{13}C NMR data on various models and respective T_1 values. Semi-synthetic tetracyclines e.g. lymecycline and rolitetracycline were also discussed. Chapter 4 is devoted to the discussion of NMR features of the degradation products of tetracyclines (^{1}H and ^{13}C NMR). A section was also included on the quantitative investigation of tetracyclines by ^{13}C NMR. Progesterone was chosen as the reference compound. Relaxation agents which are claimed to reduce the relaxation times of carbons were also used but no consistent results were obtained.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1) An analytical method was developed using PRP-1 packing material, the mobile phase consisting of tetrahydrofuran (THF), acetonitrile (ACN), propan-2-ol and buffer.

Initiall one organic solvent was used at a time in varying proportions, and the percentage composition which resulted in kappa values between 1-10 was selected. These three percentage compositions were blended in various proportions and the chromatographic results studied with respect to resolution, peak symmetry, width at half height and total time taken to analyse a mixture of tetracyclines. The influence of pH on the resolution of the mixture was studied using system 5 and the basic parameters responsible for the elution order of tetracyclines are discussed.

2) The retention mechanisms involved between tetracyclines and PRP-1 material were investigated at pH 7.3. At this pH, the tetracycline molecule exists predominantly as a net anion, so that the employment of a cationic pairing-ion should influence the degree of retention. Retention was found to be affected by the number of carbons in the non-polar moiety of the pairing-ion i.e. a dodecyltrimethylammonium salt produced longer retention than a trimethylammonium salt.

The presence of counter-ions e.g. Cl^-, Br^- and F^- etc. also affected the retention to a significant degree. The retention values were analysed statistically. The concept of adsorption was also investigated.

3) Degradation of tetracycline and oxytetracycline were investigated. Solutions of known concentration were prepared at various pH values ranging from 1-11. The samples were injected on to the column at day 0 and at regular intervals thereafter. Peak height and peak areas were determined. Rate constants and half life periods were calculated. Since TC and 4-epi TC eluted simultaneously, the strength of the mobile phase had to be reduced to 25% organic modifier to achieve resolution. The rate of formation of 4-epi TC was then calculated. Degradation of TC under various physical conditions of light, temperature etc was also investigated.

4) a: Semi-preparative HPLC

A PRP-S 25 cm column was used with the optimum mobile phase to separate one of the degradation products of oxytetracycline.

b: Comparison of PRP-1 and PLRP-S columns

The PRP-1 and PLRP-S packing materials are very similar in physical characteristics, except the average particle size of PLRP-S is less than 10um. This would increase the total surface area of the PLRP-S material and it was hoped that a resolution might be achieved between TC and OTC which normally eluted simultaneously. Resolution with the PLRP-S column was marginally better (OTC and TC were 40% resolved) due to improved column efficiency, without increasing the time required for analysis.

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CHAPTER 2

^{1}H NUCLEAR MAGNETIC RESONANCE

CHAPTER 3

^{13}C NUCLEAR MAGNETIC RESONANCE

CHAPTER 5

STANDARDS AND REFERENCE COMPOUNDS

CHAPTER 6

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Section 1

Solvent optimisation with four solvents to analyse tetracyclines

CHAPTER 1

INTRODUCTION

Section 1

1.1 General background and history

1.2 Nomenclature, absolute stereochemistry and numbering system

1.3 Pharmaceutical considerations

- 1.3.1 General
- 1.3.2 Dosage
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- 1.3.4 Mechanism of action

1.4 Degradation of tetracyclines

- 1.4.1 Reversible epimerization at C_4
- 1.4.2 Dehydration at $C_{5a,6}$
- 1.4.3 Phthalide formation in base

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INTRODUCTION

The tetracycline antibiotics first appeared in clinical practice in the late 1940s. Because of their wide spectrum of activity, convenience of administration and relative lack of dangerous side effects, they quickly assumed a major role in chemotherapy. At present, several hundreds of derivatives have been reported in the literature and several semi-synthetic analogs are in clinical use. All of the major fermentation derived tetracyclines have now been prepared by total chemical synthesis, although not yet in economically viable yields.

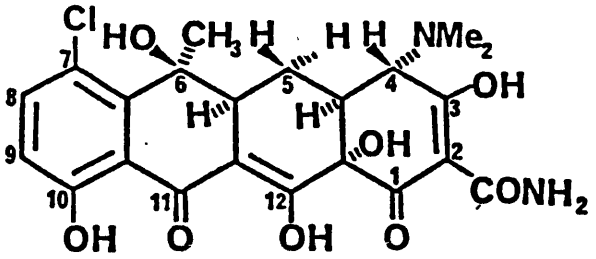
This thesis is concerned with the analytical problems associated with their use i.e. specific identification of the main antibiotics and detection of their degradation products, and the evaluation of quantitative methods with specific reference to nuclear magnetic resonance (NMR) and high pressure liquid chromatography (HPLC) procedures. The precise stereochemistry of some of the compounds is also investigated. A brief introduction to this important group of chemotherapeutic agents is now presented.

1.1 GENERAL BACKGROUND AND HISTORY

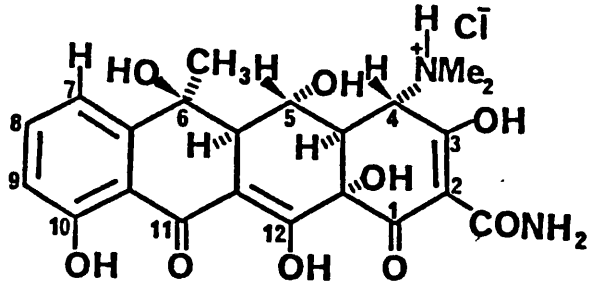
Tetracyclines are a group of broad spectrum antibiotics which have been used for over 30 years in the treatment of both human and animal diseases and also as feed additives to promote the growth of live

stock. Chlortetracycline (1, CTC, Trade name: Aureomycin) was the first member of the tetracycline family to be discovered. The tetracycline era was inaugurated by an announcement on 21st July 1948 by D.M. Dugger at a meeting of the New York Acadmey of Sciences. CTC was discovered through a soil screening programme established at the Lederle laboratories. The producing culture was named Streptomyces aureofacies. CTC was characterised by a broad activity spectrum against pathogenic micro-organisms, oral activity and relative freedom from toxicity, so it rapidly earned a major place in clinical medicine. A year later, Pfizer laboratories reported the discovery of oxytetracycline (2, OTC, Trade name: Terramycin) in fermentation liquors of Streptomyces rimosus (Regna et al 1951). During the course of experiments, directed at elucidation of the structure of OTC and CTC, it was discovered that hydrogenation of CTC resulted in halogenolysis and that the product tetracycline (3, TC Trade name: Achromycin) retained the useful activity spectrum of the first two members of the family (Boothe et. al. 1953). It was also more stable than OTC and CTC. Subsequent to these reports, TC was found to be present in fermentations of both S. aurefaciens and S. rimosus, as well as strains named S. viridofaciens.

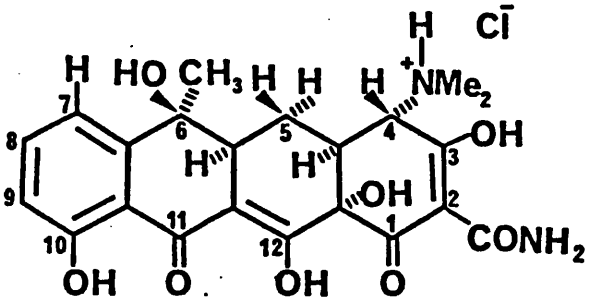
In 1957, studies with mutant strains of S. aurefaciens resulted in the formation of a new, highly active antibiotic lacking the 6 - methyly group of CTC (Table. 1)(McCormick et al. 1957). The new antibiotic, 6-demethylchlortetracycline (6-demethyl CTC, 4) was



(1) CTC HCl



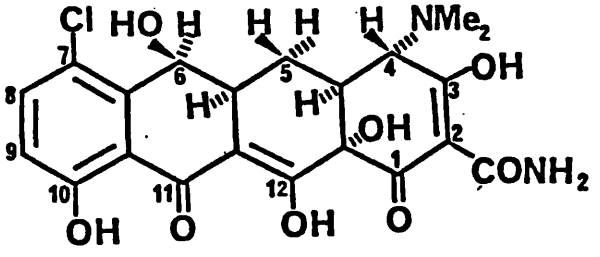
(2) OTC HCl



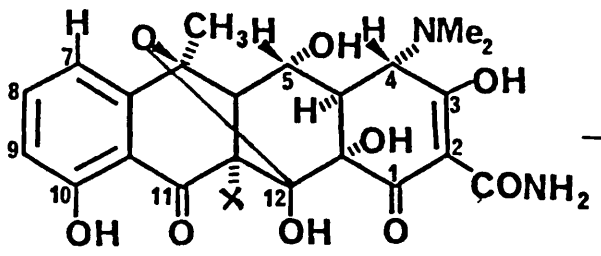
(3) TC HCl

considerably more stable than its predecessors. The higher and more sustained blood levels obtained with this drug allowed the administration of smaller doses at less frequent intervals while the antimicrobial activity remained the same. In addition to to its clinical use, 6-demethyl CTC has been the raw material from which many new and interesting derivatives have been made. A number of other tetracyclines have been produced by isolation from mutant strains of S. aureofaciens, S. rimosus or by chemical modification of the existing antibiotics. Those of commercial interest are 5-hydroxy-6-methylene TC (methacycline (5), 6-deoxy-OTC (doxycycline (6) and pyrrolidinomethy TC (rolitetracycline).

In 1965, with the discovery that C_{11a}-halogenation -dehalogenation constituted a valuable protection scheme, methacycline was prepared by selective dehydration of a suitably protected analog of OTC (7) followed by the removal of the protecting group (see page 6).



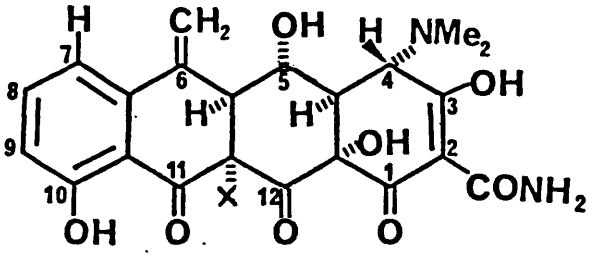
(4) 6-demethyl CTC



OTC analogue (7)

(X = Cl)

$-H_2O$



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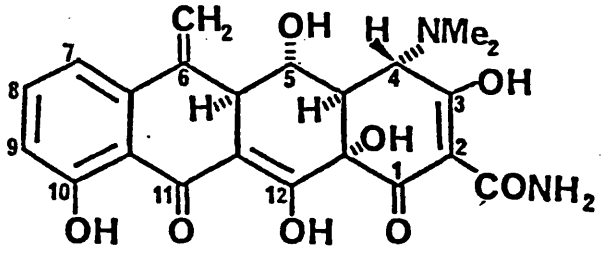
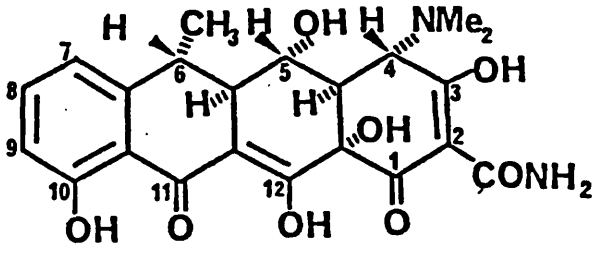


Methacycline (5)

[H]



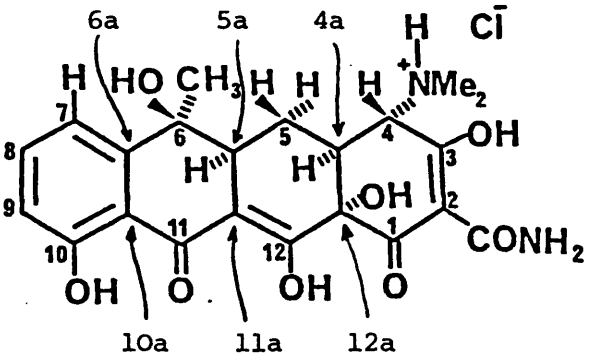
Doxycycline (6)

Methacycline was introduced commercially by Pfizer (Blackwood et al 1963). Shortly thereafter, in 1967, one of the hydrogenated products of methacycline, 6-deoxy OTC (6, doxycycline) was released (Stephen et al. 1963). Both doxycycline and methacycline are more lipid soluble than OTC. Doxycycline has the virtue of requiring lower doses on oral administration than its predecessors, and its slow excretion allows it, under some circumstances, to be the first "one a day" tetracycline. The most recently introduced member of the tetracycline family (1972) is 7-dimethylamino-6-demethyl-6-deoxy TC (Minocycline), a substance derived from the chemical transformation of 6-demethyl CTC. This drug has the broadest spectrum of any tetracycline now in use since it is active against a number of strains which are resistant to other tetracyclines.

1.2 NOMENCLATURE, ABSOLUTE STEREOCHEMISTRY AND NUMBERING SYSTEM

All of the tetracyclines are related by the common perhydronaphthacene framework. The structural formula (3), is that of tetracycline HCl and is correct in an absolute stereochemical sense. The rings are lettered A through D from right to left and the numbering starts at the bottom of ring A.

The chemical abstract formal indexing names for these antibiotics are very complex and are rarely used. The official name for tetracycline is:-



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(3) Tetracycline HCL

Tetracycline:2-naphthacenecarboxamide,4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-[60-54-8]4S-4α,4aα,5aα,6β,12aα.

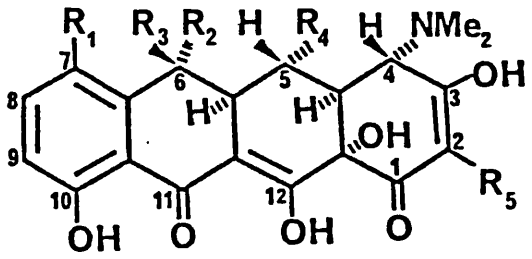
For convenience, trivial names are often used which are based on the structural formula (3). For example, CTC can be unambiguously designated as 7-chlorotetracycline, OTC as 5-hydroxytetracycline and so on. Structures of various tetracyclines are listed in Table 1.

1.3 PHARMACEUTICAL CONSIDERATIONS

1.3.1 GENERAL

The tetracyclines are effective against a wide variety of aerobic and anaerobic gram-positive and gram-negative bacteria (Mitscher, 1978). However, many strains of bacteria e.g. streptococci, staphlococci and gonococci have been shown to be resistant to the tetracyclines.

Table. 1 Structural formulas of the various members of the tetracycline group of antibiotics.



Name	Abbr.	R_1	R_2	R_3	R_4	R_5
Tetracycline	TC	H	CH_3	OH	H	$CONH_2$
Chlortetracycline	CTC	Cl	CH_3	OH	H	$CONH_2$
6-demethyl CTC HCl		Cl	H	OH	H	$CONH_2$
Minocycline		NMe_2	H	H	H	$CONH_2$
Oxytetracycline	OTC	H	CH_3	OH	OH	$CONH_2$
6-Deoxy OTC		H	CH_3	H	OH	$CONH_2$
Methacycline		H	$=CH_2$		OH	$CONH_2$
2-Cyano TC		H	CH_3	OH	H	CN
Lymecycline		complex of TC with CH_2O and lysine				
Rolitetracycline		H	CH_3	OH	H	$CONHCH_2NC_4H_8$

Therefore culture and sensitivity testing is recommended if the infecting organism is susceptible to the tetracyclines. In general, if a micro-organism is resistant to one of the tetracyclines it is usually resistant to the entire family. The tetracyclines are bactereostatic rather than bacteriocidal unless large doses are used (Mitscher, 1978).

1.3.2 DOSAGE

The tetracyclines are usually effective at extremely low concentrations, of the order of 1ug/ml. Although, oral doses of tetracyclines are not completely absorbed from the gastro-intestinal tract, and adsorption may be hindered by food, milk and antacids, oral doses are still the favoured dosage forms.

1.3.3 SIDE EFFECTS

For the most part, the side effects commonly encountered in clinical practice with the tetracyclines are annoying or uncomfortable rather than life threating. Anorexia, vomiting, nausea, heartburn, flatulence and diarrhoea apparently occur in about 10% of the patients recieving 2g or more of TC daily (Martindale 27th edition). Another side effect observed is the discolouration of the teeth in children receiving a tetracycline. A tetracycline-calcium

orthophosphate complex, which is slowly oxidised by light from yellow to brown, is believed to be responsible. Tetracycline is not prescribed for pregnant mothers because tetracycline can cross the placental barrier and appear in mother's milk. Moderate doses can lead to a definite yellowing, a reversible slowing of bone growth in the foetus, and infants and young children below the age of eight years when the teeth and bones are undergoing rapid development (Ibsen et al. 1965). Dispensing of out of date tetracyclines have led to the reversible Fanconi syndrome, characterised by anorexia, vomiting, weight loss and rash. These symptoms are believed to be caused by a degradation product of TC, anhydrotetracycline (anhydro TC)(Mull, 1966). Expiry dates should therefore be respected, and tetracyclines should not be stored under conditions of excessive heat and humidity.

1.3.4 MECHANISM OF ACTION

The tetracyclines are actively transported into the cells of susceptible bacteria and exert a bacteriostatic effect by inhibiting protein biosynthesis after binding to the ribosomal sub-particle (Pratt, 1973). The ribosomes of man are less sensitive to the effect of tetracyclines. This accounts for their useful selective toxicity. Other relavant information regarding the mechanism of action, interference with the host's natural defences etc., which are beyond the scope of this thesis, have been reported (Mitscher, 1978).

1.4 DEGRADATION OF TETRACYCLINES

At least seven of the tetracyclines or their derivatives are now in regular clinical use as antibiotic agents (Table 2), hence the problems of quality control relating to the pure antibiotic compounds and to the pharmaceutical formulations, developed for oral and parental use, are of acute significance to pharmaceutical industry. Although, most of the tetracyclines differ in various ways, the properties of all the compounds are dominated by the common tetracycline nucleus (Fig. 1) and the array of functional groups.

Table 2. In-vitro potency of the major tetracyclines compared to TC (Blackwood and English, 1970).

Tetracycline	Kl. pneumoniae	S. aureus 209P
TC	1.0	1.0
CTC	2.5	3.5
OTC	1.0	0.8
6-demethyl CTC	---	3.0
Doxycycline	1.4	---
Methacycline	2.3	---

Hence, the physical properties, notably the colour, appearence, solubility and many spectroscopic features of the tetracyclines are similar and make difficult the specific identification of the

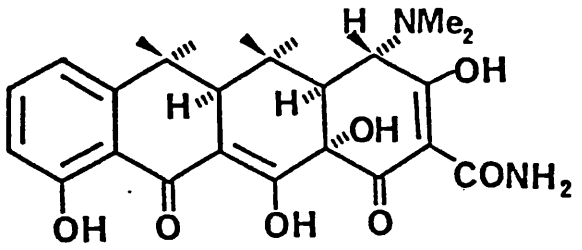


Fig. 1 Common tetracycline nucleus and functional groups

individual members. Analytical problems of this type are compounded by the fact that common degradation products of the antibiotics share many of the properties of the parent compound. Since much work of this thesis is involved with the analytical problems of the degradation contaminants, it is advisable to present a brief discussion of the various mechanisms and sites involved in the degradation processes.

1.4.1 REVERSIBLE EPIMERISATION AT C_4

The dimethylamino function at C_4 of all bioactive tetracyclines is positioned 1,3-diaxially eclipsed with respect to the C_{12a}-OH group in acidic solutions (see Fig. 2a). The process of epimersation relieves this interaction and takes place via enol (11) form (Doerschuk et al. 1955). The product, 4-epi TC, has no significant antibacterial activity. The reaction takes place more readilly at pH 3-5 in aqueous solutions and is a reversible first order reaction (see kinetics study). In alkaline solutions, using precise

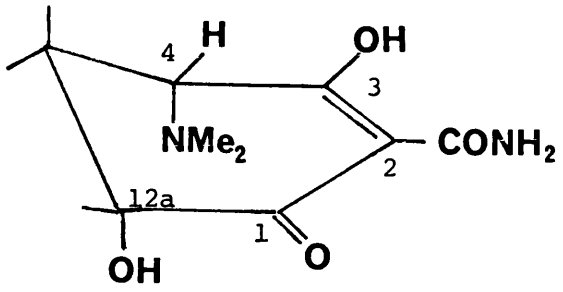


Fig. 2a Stereochemistry at C_{4a} and C_{12a}

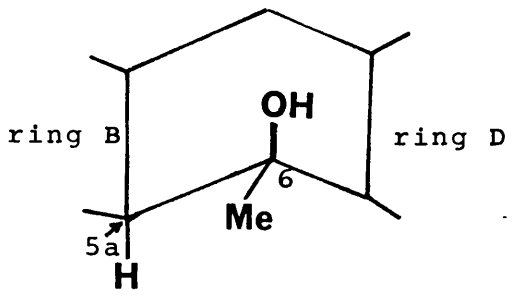
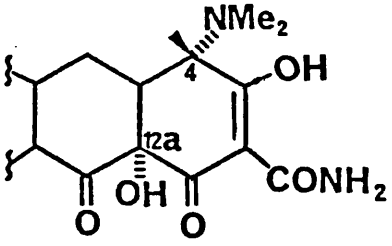


Fig. 2b Stereochemistry at C_6

conditions, 4-epi TC is converted almost completely back to the bioactive tetracycline in the presence of chelating metals (Korst et al. 1968). This has been explained as being due to the complexing metals "tying" together the C_4-dimethyamino and C_{12a}-hydroxy functions, thus overcoming their steric repulsions.



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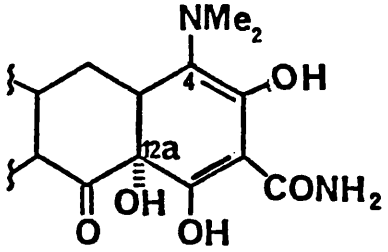

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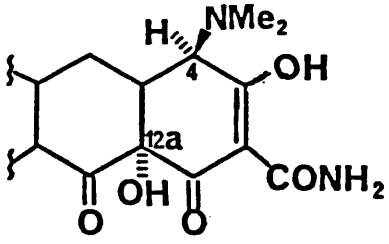
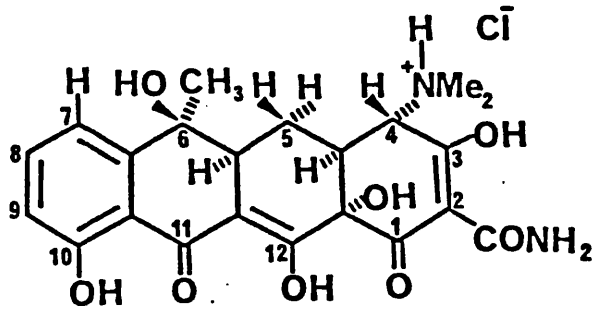


(12) 4-epi TC (partial structure)

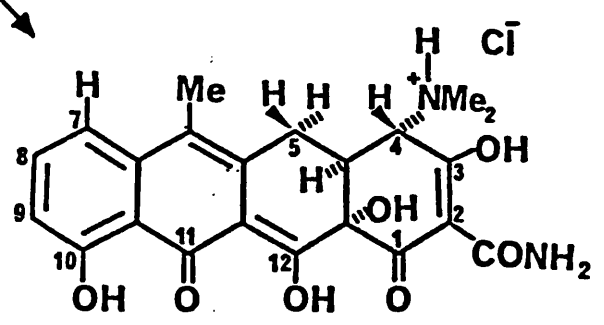
1.4.2 DEHYDRATION AT $C_{5a,6}$

The C_6-OH group is axial and antiperiplanar trans to the C_{5a}-H (see Fig.2b). Therefore it is sterically ideal for an E_2 reaction. In tetracyclines, the reaction is assumed to proceed via (13)leading to the formation of Anhydro TC (14) in which the ring C becomes aromatic

(see NMR evidence in Chapter 5). The anhydro TC possess invitro activity but has not found clinical application. The anhydro formation takes place progressively at pH values lower than 2.0, especially on heating. 6-Demethyl CTC has a secondary OH group at C_6 and so it is more stable to acid dehydration (McCormick et al. 1957). Minocycline lacks an -OH group at C_5 or C_6 and so can not degrade in this manner. OTC dehydrates in the same manner as TC, but anhydro OTC (15) is very unstable under the reaction conditions, undergoing opening of the ring B mediated through the C_5-OH group to produce the isomeric phthalides, α and β apo OTC (16).



TC HCl (3)



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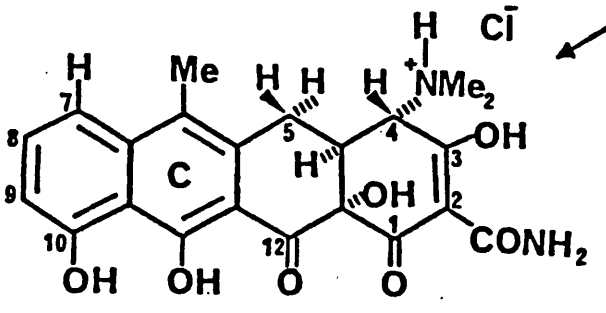
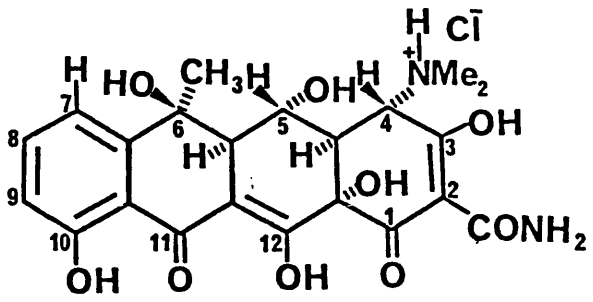
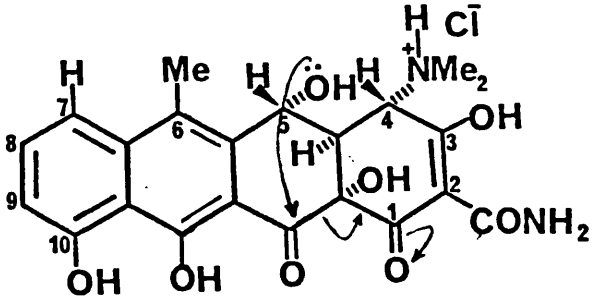


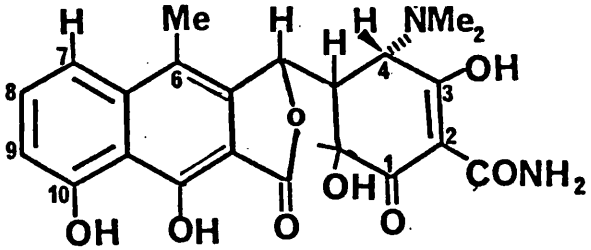
Anhydro TC HCl (14)



OTC (2)



(15)

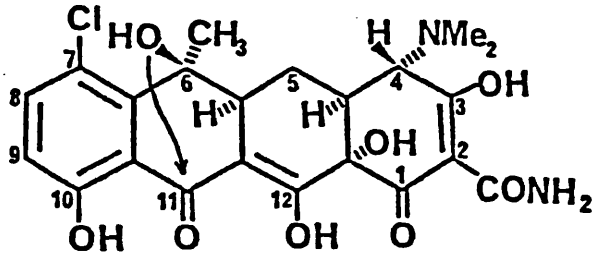


(16)

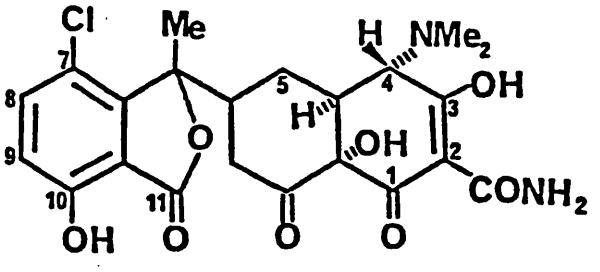
α and β -apo OTC

1.4.3 PHTHALIDE FORMATION IN BASE

In alkaline solutions, tetracyclines having a C_6-OH cleave readily to form iso-tetracyclines. CTC seems to be specially prone to this type of degradation (Waller et al. 1952). The mechanism is presumed to be as follows:



CTC (1)



(17)

Iso CTC

1.5 AIMS AND OBJECTS

It is clear from the preceeding brief survey of tetracyclines that this group of closely related compounds presents an analytical challenge that can not be met adequately by currently employed techniques.

Effective methods are required for :

1) The specific identification of individual members.

2) The detection of degradation products.

3)Quantitative procedures for determining the antibiotics both in raw drug state and in pharmaceutical preparations.

The primary analytical technique chosen to meet these problems is high performance liquid chromatography (HPLC), a method of high sensitivity and potentially capable of high resolution. HPLC. may often differentiate compounds of very similar structures, even diastereoisomers (also antipodes when chiral columns are used). The technique provides accurate information on the number of components and their relative amounts in an analyte. To identify the various components chromatographic data on standards are required. Thus an important adjunct to the development of HPLC procedures is the availability of reference compounds of authenticated structure. In this case, standard tetracycline antibiotics and their degradation products are required. Because of the close structure similarity of this group of compounds, NMR spectroscopy is considered the most effective method for achieving characterisation of the standards, and

the first part of the thesis describes the NMR investigations based upon both proton (^{1}H) and carbon-13 (^{13}C) magnetic nuclei.

Section 2 SPECTROSCOPIC ANALYTICAL METHODS

2.1.1 IR

2.1.2 UV/VIS

2.1.3 Spectroflurimetry

2.1.4 X-Ray crystal analysis

2.1.5 Miscellaneous spectroscopic methods

ANALYTICAL METHODS

2.2.1 Microbiological methods

2.2.2 Non-aqueous titrations

2.2.3 Polarography

CHROMATOGRAPHIC METHODS

2.3.1 Paper chromatography

2.3.2 Thin layer chromatography

2.3.3 Column chromatography

SECTION 2

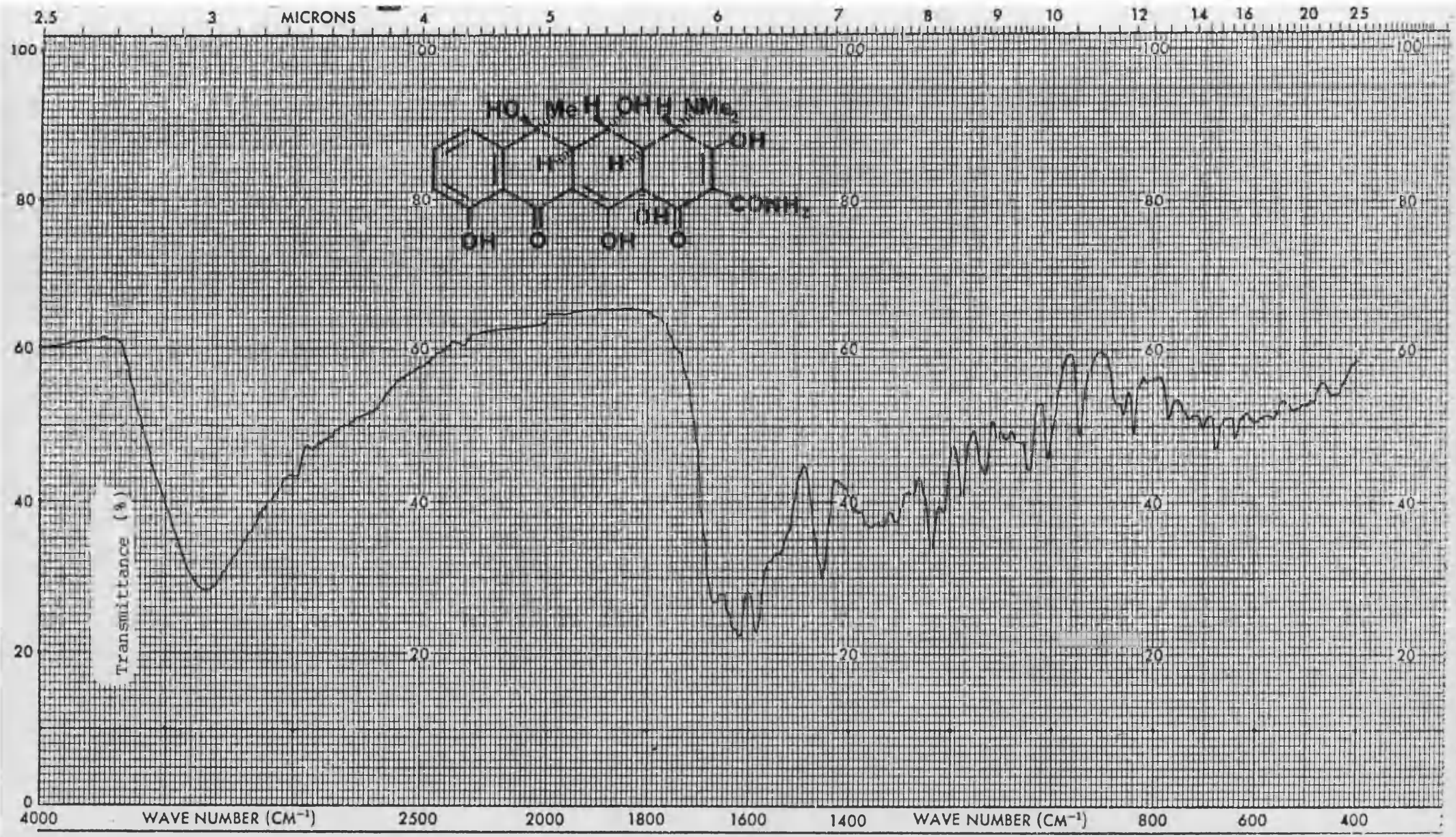
2.1 SPECTROSCOPIC ANALYTICAL METHODS

Spectroscopy plays a major role in the structural analysis of tetracyclines. Although this thesis is mainly concerned with NMR spectroscopy and HPLC, it was considered that a brief account of other spectroscopic and non-spectroscopic techniques be included to demonstrate the comparative value of other approaches.

2.1.1. INFRA-RED (VIBRATIONAL) SPECTROSCOPY

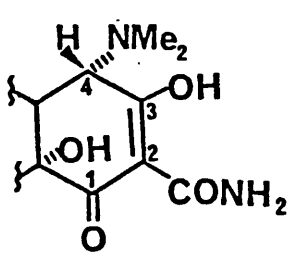
Due to the limited solubility of the analyte in conventional solvents, the IR spectra of tetracyclines are normally determined as solids using KBr disc or Nujol mull techniques.

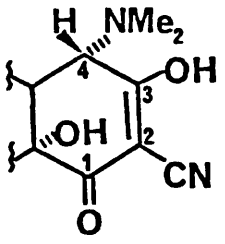
The carbonyl region (1620-1800 cm^{-1}) is of interest, but several of the carbonyl groups of the tetracycline molecule are amide in character, conjugated with double bonds or strongly hydrogen bonded. Hence, the carbonyl region is almost identical for most of the tetracyclines. The main difference therefore lies in the region below 1000 cm^{-1} known as the "finger print" region. The IR spectrum of oxytetracycline HCl is shown in Spectrum 1. In certain cases, appearence of specific absorption bands in the IR spectrum is a valuable tool for identification. For example, during the preparation of the 2-cyano derivative of OTC (18), the presence of an



Spectrum 1. IR of oxytetracycline hydrochloride using KBr disc. (not calibrated)

absorption band at 2200 cm^{-1} (absent in OTC), due to -C≡N,



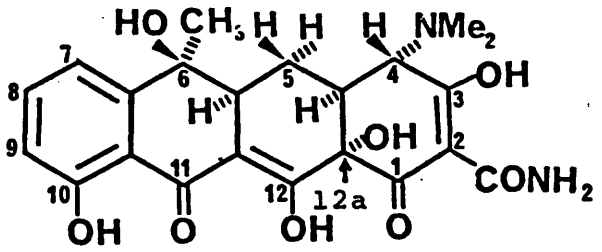


OTC (2) (partial structures) 2-cyano OTC (18)

signalled the success of the synthetic process (Spectrum 2).

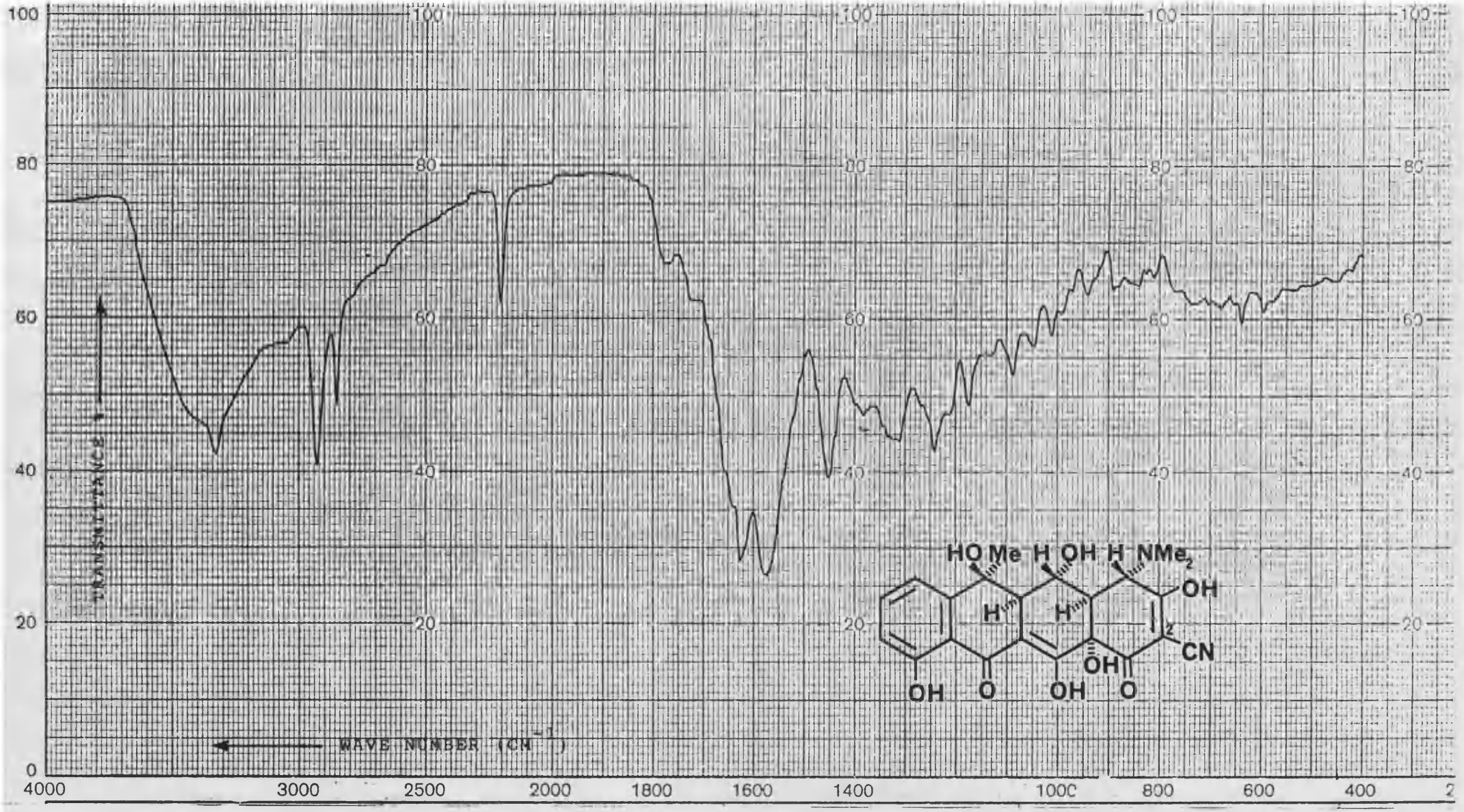
2.1.2. ULTRA-VIOLET (ELECTRONIC) SPECTROSCOPY (UV)

Tetracyclines possess two distinct chromophores which are conventiently divided by C_{12a} (3).



ring A

(3)

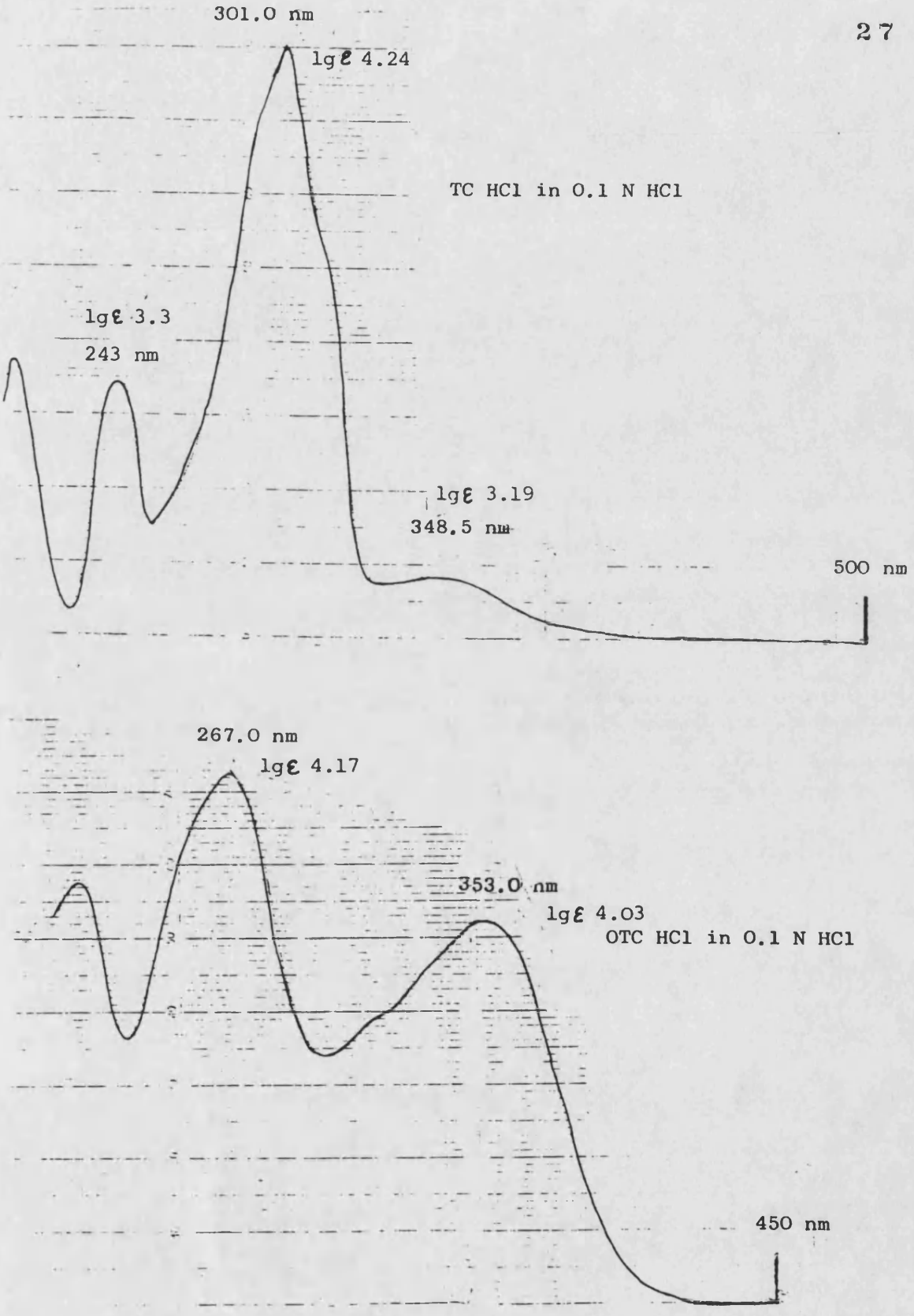


Spectrum 2. IR spectrum of 2-cyano OTC (KBr disc, not calibrated)

The tricarbonyl system of ring **A** contributes a strong absorption band at about 260 nm, while the aromatic ring **D** conjugates with the diketone moiety in rings **B** and **C**, to produce a visible band at 349 nm. The UV-VIS spectra of OTC and TC are shown in Spectrum 3. The UV-VIS data for various tetracyclines is recorded in Table 3. Due to the fact that differences between the UV/VIS spectra of tetracyclines are minor, electronic spectroscopy on its own is not suitable for differential analysis of tetracyclines. Confirmation of the identity of a particular tetracycline can be achieved by spectral comparison with that of the authentic material but care is necessary. Dehydration at C_{5a-6}, results in the ring **C** becoming aromatic. This produces a significant change in the UV/VIS spectrum. The ratio of the peeks at 348.5 nm (TC HCl) and 430 nm (anhydro TC HCl) provides an assay for the presence of these two drugs in the mixture. A simple procedure for CTC was developed by Levine et al. (1949), in which the antibiotic is dehydrated by heating in acid and the resulting solution (mainly the anhydro CTC) is examined spectroscopically at 440 nm. This is preferred to direct examination of CTC at 367 nm because there are fewer interferences at longer wavelengths. The same method may be applied to TC HCl.

2.1.3. SPECTROFLUORIMETRY

CTC HCl produces an intensely fluorescent solution when heated at pH 7.5 in aqueous buffer (Levine et al. 1949). This method has been used to check antibiotic levels in animal feeds. Advantage was taken



Spectrum 3. UV spectra of TC HCl and OTC HCl in 0.1 N HCl.

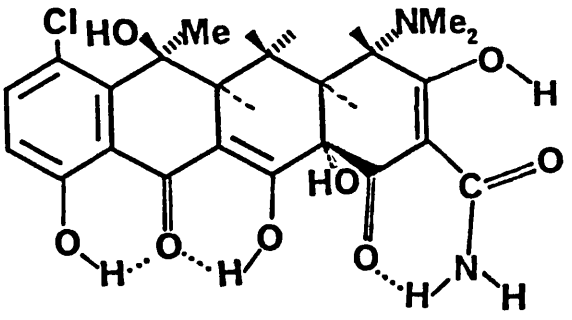
Table 3 UV/VIS spectra of various tetracyclines.

Tetracycline	solvent	UV/VIS maximas
TC HCl	0.1 N HCl	348.5, 301.0, 243.0
OTC HCl	" " "	353.0, 267.0, 215.0
6-deoxy OTC	" " "	342.8, 266.5, 202.4
methacycline	" " "	340.0, 240.0, ---
6-demethyl CTC	" " "	366.0, 266.3, ---
anhydro TC	" " "	430.0, 271.0, 221.0
anhydro TC	methanol	440.0, 275.0, 224.0

of the greater lipophilicity of dehydrated TC by Kelly and co. workers (1969). TC is dehydrated by heating in acid, extracted at about pH 4-5 with chloroform, and the fluorescenece of the aluminium chelate is then measured.

2.1.4 X-RAY CRYSTAL ANALYSIS

The structures and solid state conformations of several tetracyclines have been reported. CTC HCl was examined first (Donohue et al. 1963), and the work resulted in assignment of the stereochemistry as shown below.



(19)

Subsequently, OTC HCl was also determined by this technique. One of the limitations of X-RAY crystal studies is the static nature of the measurements. One normally cannot get much information regarding solution dynamics except by inference (see section on conformation, page 75).

2.1.5 MISCELLANEOUS SPECTROSCOPIC METHODS

Solutions of tetracyclines in aq. NaOH contain a large concentration of free radicals which have been characterised by electron spin resonance methods (Lagercrantz and Yhland 1963). Cobalt and nickel ion complexes of the tetracyclines have been studied by reflectance methods leading to the conclusion that binding occurs in ring A (Baker and Brown, 1966). Magnetic moments have also been determined.

2.2 MISCELLANEOUS ANALYTICAL METHODS

2.2.1 MICROBIOLOGICAL METHODS

The potency of a sample of an antibiotic is determined by comparing the dose which inhibits the growth of a suitable susceptible micro-organism with the dose of the standard preparation of that antibiotic which produces the same degree of inhibition. The B.P. (1980) lists microbiological tests for the potency of the antibiotic, abnormal toxicity, pyrogens and sterility. These methods are non-specific and reflect the end use of the agents.

2.2.2 NON-AQUEOUS TITRATIONS

Since tetracyclines contain basic and acidic centres, they may also be determined by titrations in the non-aqueous media. Yokoyama and

Chatten (1958) have described the titration of tetracyclines in nitromethane-formic acid-benzene solvent mixture with methylene blue indicator using perchloric acid as titrant.

2.2.3 POLAROGRAPHY

Caplis et al. (1965) described a polarographic method for qualitative anallysis of TC using alternating current. It was claimed to be faster and more senstive than using direct current, and the results agreed with the microbiological assays. It was also possible to assay OTC in the presence of CTC by using the above method.

2.3 CHROMATOGRAPHIC METHODS

2.3.1 PAPER CHROMATOGRAPHY

In even the earliest studies of tetracyclines, paper chromatography methods found great utility as adjuncts to other techniques in following bio-synthesis in fermentation broths (Bird, Jr. and Pugh 1954). In most cases phosphate buffered paper was found to be necessary, detection then being carried out by UV. Many of the problems associated with paper chromatography of the tetracyclines (streaking and diffusion of spots), were rationalised by Kelly and Buyske (1960) on the basis of chelation of trace metals in the paper. They claimed much improved resolution by impregnation of the paper by

EDTA prior to spotting. This system has been adopted almost exclusively in thin layer chromatography (TLC) which has largely superceded paper chromatography.

2.3.2 THIN LAYER CHROMATOGRAPHY (TLC)

Kapodia and Rao (1964) were able to resolve TC, CTC and OTC by the use of TLC impregnated with sequestering agents (e.g. EDTA). In a series of papers, Simmons et al. (1966) have described systems capable of resolving TC from its impurities, based on layers of micro-crystalline cellulose buffered with an aqueous solution of 0.1M EDTA and 0.1% ammonium chloride. In the course of the work TLC was carried out according to methods outlined in the B.P. (1980). Several changes have to be made to the official method before any reasonable accuracy was achieved. R_f values obtained were found to be within a very narrow range. TLC was found to be a convenient method to check the purity of a sample, but streaking was often encountered suggesting that the amount of water left in the silica was critical.

2.3.3 COLUMN CHROMATOGRAPHY

Washed diatomaceous earth impregnated with buffered stationary phases containing EDTA has been used to separate and quantify tetracycline mixtures in the presence of their degradation products (Fike and Brake, 1972; Kelly, 1964). A further elaboration allowed automation

to increase the number of samples which could be processed (Ascione et al. 1972).

Section 3

3.1 Starting materials

3.2 Apparatus

3.2.1 Melting point

3.2.2 IR

3.2.3 UV/VIS

3.2.4 pH measurements

3.2.5 NMR (^{1}H 100 and 400 MHz)

NMR (^{13}C 22.5 and 67.8 MHz)

SECTION 3

3.1 STARTING MATERIALS

Details of the samples and their sources used throughout this work are given in Table 4. All of the materials were kindly donated and from the correspondence it is evident that they are all of a quality deemed satisfactory for use in various pharmaceutical preparations.

3.2 APPARATUS

3.2.1 MELTING POINTS

Melting points were recorded in 1mm Pyrex capillary tubes using a Townson and Mercer melting point apparatus.

3.2.2 INFRA-RED SPECTROSCOPY

The IR spectra were obtained on a Unicam SP200 IR spectrometer. The KBr discs were prepared by mixing 1-1.5mg of the sample with 300mg of KBr. A nujol mull was an alternative choice of recording the IR spectra.

3.2.3 UV-VIS SPECTROSCOPY

The UV-VIS spectrophotometer used was a Perkin-Elmer 550s UV-VIS instrument.

Table 4. The tetracyclines employed in the present work.

Drug	Form	Batch number	Company
TC HCl	Raw drug	12-3334	Lederle
Achromycin	"	048386/718064	"
TC HCl	"	2192291/926623	"
Achromycin	"	R7541	Lederle
OTC HCl	"	003-51827	Pfizer
OTC Ca.	"	003-50772	"
OTC $2H_2O$	"	003-53755	"
OTC $2H_2O$	"	503-53721	"
OTC HCl	"	103-51765	"
Ledermycin	"	048151-1043	Lederle
Methacycline	"	903-56701	Pfizer
Demeclocycline neutral		048160/E164	Lederle
Minocin	"	48893-G25/G17-063	Lederle
Doxycycline Hyclate	"	903-58702	Pfizer
Doxycycline Hyclate	"	103-58701	"
6-epi doxycycline	"	42-PD-186	Pfizer
Aureomycin	"	038-F-021931-0500-7	Lederle
4-epi CTC HCl	"	7662B-32-1	Cyanamid
Aureomycin	"	29373-B148N103	Lederle
Aureomycin	"	1931-05/C176	Lederle
Anhydro CTC neutral	"	7662B-43-1	"
Limecycline	"	R93E025	Cyanamid (USA)

3.2.4 pH MEASUREMENTS

Where required, pH values were measured using either the PT1-6 Universal digital pH meter or Whatman pH paper (full range 1-14). The pH meter was calibrated using solutions of known pH (in most cases pH 4.0, 6.0 and 9.2 depending on the operating conditions).

3.2.5 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

^{1}H NMR (100 MHZ)

The ^{1}H NMR spectra were recorded on Jeol PS100, operating at 100MHz, spectrometer. The samples were spun in 5mm o.d. tubes. The average concentration of the samples was 60-80 mg/0.5 ml of solvent. The probe temperature was 28^{o}C. The chemical shift data were recorded as parts per million (ppm) with tetramethysilane (TMS) as the internal standard (0 ppm).

^{1}H NMR 400MHz (University of Warwick, Dr. O. Howarth)

The ^{1}H NMR spectra were recorded on a Bruker WH-400 MHz spectrometer operating at 400 MHz. Samples (5-10 mg) were dissolved in 0.5 ml Dmso.d_6 with TMS as reference standard, and examined without degassing at temperatures over the range 22-70 ^{o}C and employing the standard conditions of 32K data points and 32 accumulations with digital resolution of 0.328 Hz per point. 270 MHz ^{1}H NMR spectra were carried out on Jeol JNM-GX270 spectrometer operating at 270 MHz.

^{13}C NMR

22.5 MHz

The ^{13}C NMR spectra were obtained on the Jeol FX 90Q high resolution spectrometers operating at 22.5 MHz. Samples were dissolved in a suitable deuterated solvent e.g. Dmso.d_6 or D_2O, the deuterium of the solvent provided the lock signal. Spectra were recorded with 8K data points. For an average spectral width of 5000 Hz a 4μs pulse, corresponding to a tilt angle of 30°, was employed with a 1.8192 s interval. Probe temperature was 23°C. All the chemical shifts are quoted with respect to TMS = 0 ppm.

67.8 MHz

The spectra were obtained on the Jeol JNM-GX270 spectrometer operating at 67.8 MHz. The probe temperature was 20°C. The deuterium of the solvent provided the lock signal. All the chemical shifts are quoted with respect to TMS = 0 ppm.

3.3 CHEMICAL SYNTHESIS

3.3.1 PREPARATION OF α AND β APO-OXYTETRACYCLINE

The synthesis of these isomers was carried out using the method of Hochstein et al. (1953), although several changes were made.

10g of OTC.HCl were dissolved in 0.5N HCl (20ml) and heated in a water bath at 60°C for 19 hrs. The clear dark solution which resulted was diluted to 75ml with water and adjusted to pH 3.5 with 10% NaOH. The thick yellow suspension was filtered. The solid (amorphous α and β apo-OTC) was washed thoroughly with distilled water and dissolved in hot ethanol. The α isomer precipitated and was collected after 24 hrs. The filtrate was reserved for isolation of β apo-OTC.

α -apo OTC

The solid was suspended in 10 ml of H_2O and dissolved by adding 12N HCl dropwise until pH was 1.0. The solution was concentrated in vacuo, but no precipitate appeared after 24 hrs. The solution was evaporated to dryness and the residual solid was stirred with hot ethanol for 30 min. The solution was allowed to cool. The precipitate which formed was collected and dissolved in H_2O, and then 10% NaOH added dropwise until the pH was 3.5. The precipitate which resulted was filtered and dried in a vacuum desiccator . The solid was refluxed with hot absolute alcohol for 10 min. and filtered hot. The solid was collected and dried. An IR spectrum of the sample displayed a broad band near 1750 cm^{-1} (absent in the IR spectrum of

OTC) which is due to the lactone group.

Yield 1.5g, m.p. 195-210 °C (Hochstein et al. 1953 give 190-200 °C)

β apo-OTC

The filtrate from the first alcohol crystallisation of α apo-OTC, was concentrated in vacuo to approximately 20ml, acidified with 12ml of 2.5N HCl and cooled to 5 °C for 24 hrs. 10% NaOH solution was added until pH was 3.5. The precipitae was filtered and dried in a desiccator. An IR spectrum of the sample revealed a broad band at 1750 cm^{-1} which is due to the lactone group.

Yield 3.5g, m.p. 195-200 °C (Hochestein et al. 1953 give 195-205 °C)

3.3.2 ANHYDRO TETRACYCLINE HYDROCHLORIDE

The synthesis of anhydro TC from TC followed the method of Newman (1975). 2g of TC HCl was dissolved in a boiling mixture (100ml) of isopropanol:methanol:conc. HCl in the ratios 4:1:2 respectively, and heated under reflux for 30 min. On cooling a precipitate formed which was collected by filtration and washed with isopropanol. The sample was recrystalised from methanol-conc. HCl (30:1 v/v) mixture. The bright yellow crystals were collected and dried in a low heat oven at 60 °C.

Yield 0.92g, m.p. 223 °C (Newman 1975 gives 220 °C)

3.3.3 TETRACYCLINE METHIODIDE

The synthesis of tetracycline methiodide followed the method of McCormick et al. (1957).

5g of TC base was stirred with 18ml of iodomethane in 90ml of tetrahydrofuran (THF) for 24 hrs. at room temperature. The light yellow solid, which formed, was filtered and washed with 50ml THF and then dried in vacuo at 40 °C. The solid was recrystallised from warm (50 °C) ethanol (95%). An IR spectrum of the sample displayed a broad band near 3000 cm^{-1} due to the quaternary ammonium group.
Yield 3g, m.p. 179-181 °C (McCormick et al. 1957 give 178-180 °C)

3.3.4 5,12a DIACETYLOXYTETRACYCLINE

2g of OTC $2H_2O$ base was dissolved in 40 ml dioxane and made up to 200 ml with acetic anhydride. The mixture was kept at room temperature for 14 days. The solution was evaporated to dryness under vacuum and water bath temperature below 35 °C. The yellow solid that remained was recrystallised from toluene as follows:

The solid was stirred withe toluene and heated. The insoluble precipitate was decanted and stirred with hot toluene once again. After cooling the insoluble precipitate was filtered and stirred with ether for 15 min. The ether fraction was collected. The ether fraction was evaporated to dryness. An IR spectrum of the sample displayed a resonance near 1750 cm^{-1} due to the ester

($OCOCH_3$) along with a resonance at 1720 cm^{-1} due to the ether carbonyl functions.

Yield 800mg, m.p. 210-214 °C (Hochstein et al. 1953 give 208-213 °C)

3.3.5 ISO-CHLORTETRACYCLINE HYDROCHLORIDE

2g of CTC HCl in 20 ml of 0.1N NaOH was allowed to remain at room temperature for 36 hrs. (pH of solution was 9.0). The pH of the filtrate was adjusted to 7.0. The gelatinous precipitate which resulted was slurry-washed with water and dried in a vacuum oven over conc. sulphuric acid. The precipitate was refluxed with hot methanol twice and the mixture filtered hot. On standing the filtrate produced fine crystals.

Yield 900mg, m.p. 220-225 °C (McCormick et al. 1957 give 213-224 °C)

3.3.6 2-CARBOXAMIDODIMEDONE

10g of potassium cyanate in 30 ml of water was added dropwise, dusring a period of 30 min with stirring, to a solution of 10g of dimedone in 100 ml of dimethylformamide at 100°C. the mixture was heated for another 30 min at 100°C, and was then acidified with 1N HCl acid and diluted with 60 ml of water. The mixture was cooled in an ice bath for 2 hr and the crystalline precipitate was filtered off, washed with water and dried in a desiccator. Recrystallisation

was carried out once from ether-petroleum ether mixture.

Yield 6g, m.p. 147°C (Muxfeldt et al. 1966 give 148-149°C).

The ^{13}C NMR assignments are discussed in Chapter 5

CHAPTER 2

^{1}H NUCLEAR MAGNETIC RESONANCE

^{1}H NMR

Aromatic protons of ring D

C_6-methyl protons

Dimethylamino protons

Acidic protons

Methine (4H, 4a-H, 5-H and 5a-H) and 5-H_2 methylene protons

High resolution spectra recorded above 220 MHz

Evidence of conformation

OTC HCl

Doxycycline and 6-epi doxycycline

Meclocycline and methacycline

Minocycline

6-demethyl CTC

α and β apo OTC bases

^{1}H-NMR

This account of the ^{1}H NMR features of the tetracycline antibiotics and derived compounds relates chiefly to the hydrochloride salts, since these are the forms in which these materials are usually isolated and used clinically. Basic forms of the drugs are discussed later.

For solubility purposes, the principal solvent used was deuterated dimethylsulphoxide (Dmso.d_6) with tetramethylsilane (TMS) as the reference standard (see experimental section). Such spectra include a solvent signal near 2.5ppm (a narrow multiplet due to residual hydrogen, and occasionally a water signal of variable position if the solvent has been exposed to moisture).

With the exception of the methine signals, most features of the tetracycline spectra are well resolved and readily assigned at, what are regarded today as, low operating frequencies (60 and 100 MHz). It is convenient to discuss each feature separately and to summarize the data for the whole group. Results of spectra recorded at higher frequencies (220, 270 and 400 MHz) are included where appropriate, but described in more detail elsewhere.

The different types of protons of the tetracycline antibiotics may be categorised into the following groups:-

1) Aromatic protons of the ring D

2) Methyl protons (C_6methyl and NMe_2)

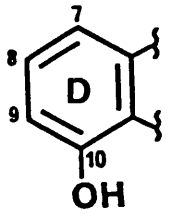
3) Acidic protons

4) Methine (4-H, 4a-H, 5-H, 5a-H) and methylene (5-H_2) protons (also vinylic (CH) protons of methacycline and meclocycline).

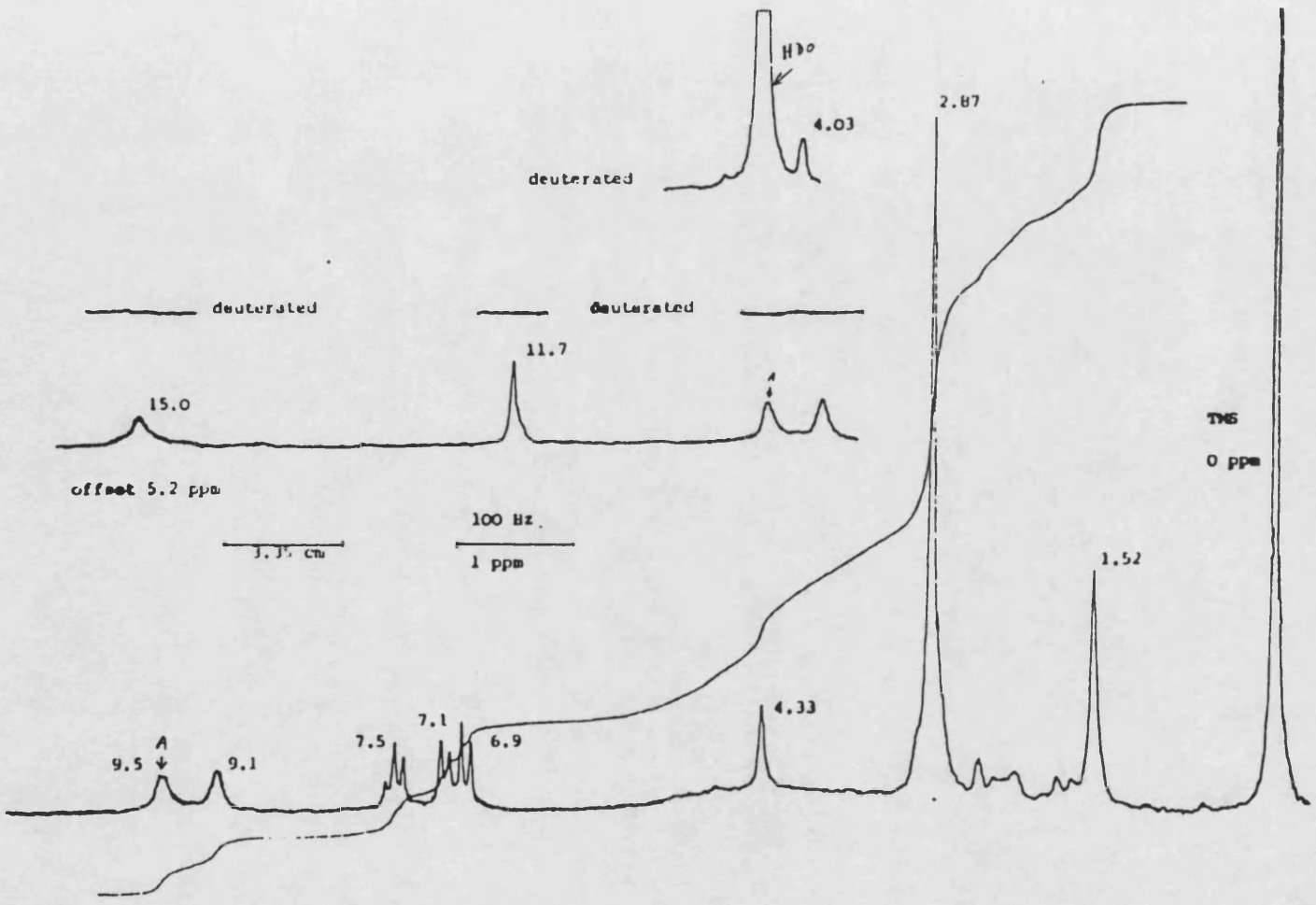
A case is now presented for the assignments of the above groups of protons, with the object of assessing the value of ^{1}H NMR spectra for the differentiation of the various tetracycline derivatives.

AROMATIC PROTONS OF RING D

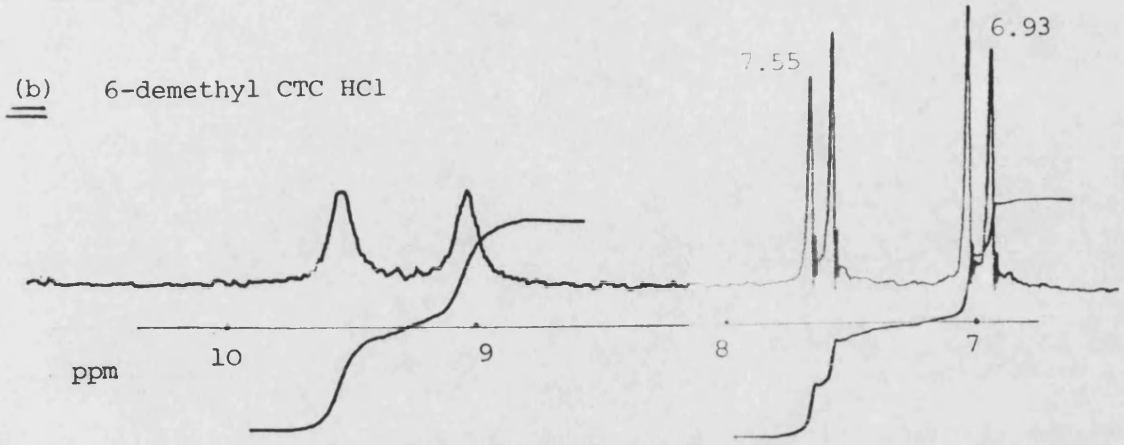
These resonances fall in the region 6.8 to 7.8 ppm. Spectra of derivatives without a substituent in ring D, apart from the C-10 hydroxy which is common to all, show a 3-proton aromatic signal composed of a broad triplet (low field due to 8-H) and a pair of doublets due to

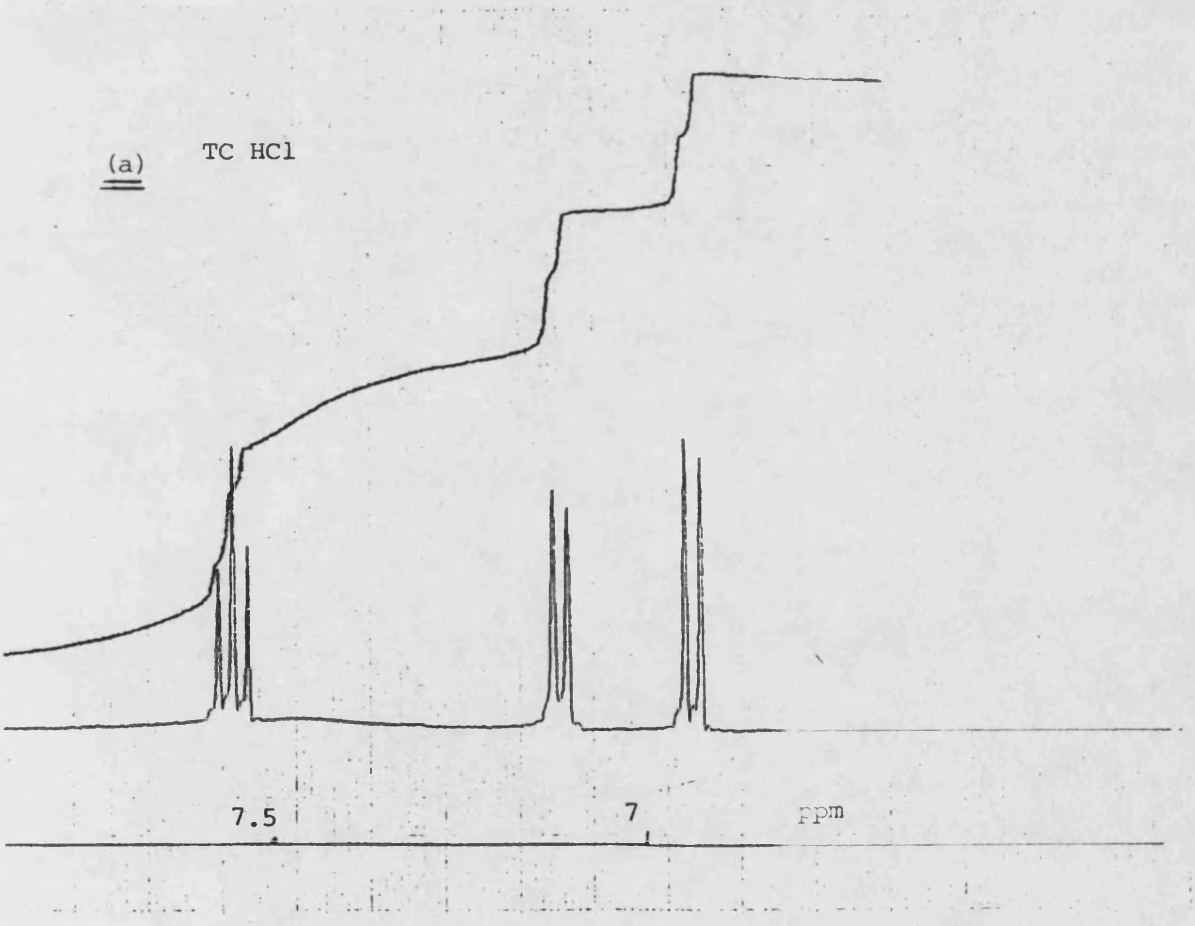


7-H and 9-H (see spectra 4 and 5a). The 8-H resonance is lowest field due to the known shielding influence of phenolic-OH at ortho and para positions (Stothers, 1972). In contrast, and of analytical utility, spectra of derivatives with a 7-substituent display a typical AB doublet pair (Spectrum 5b). The resonances of ring D are listed in Table 5.



Spectrum 4. The spectrum of TC HCl (^{1}H NMR 100 MHz) in Dmso.d_6 /TMS





Spectrum 5. ^{1}H NMR spectra of (a) TC HCl (400 MHz) and (b) 6-demethyl-chlortetracycline HCl in Dmso.d_6/TMS

Table 5. Aromatic resonances of ring D (100 MHz) in ppm from TMS.

Solvent Dmso.d_6 (TMS = 0 ppm)

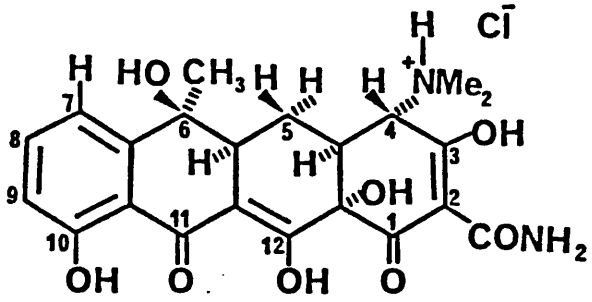
Name	Triplet*	Doublet**
TC HCl	7.5	7.1, 6.9
TC base	7.5	7.1, 6.9
OTC HCl	7.5	7.1, 6.9
CTC HCl	---	7.5, 6.9
6-demethyl CTC HCl	---	7.6, 6.9
Methacycline	7.6	7.0, 7.2
Doxycycline	7.5	6.9, 6.85
Meclocycline	---	6.95, 7.0
Minocycline	---	6.81, 7.39

* Triplet separation:- approximately 8-10 Hz

** Doublet separation:- approximately 8-10 Hz

C_6-METHYL PROTONS

The broad three proton resonance at 1.5 ppm of TC HCl (3) (Spectrum 4) is a diagnostic feature of TC derivatives with a methyl substituent at C_6 (Table 6). The broad nature of this and related resonances of other derivatives probably result from the operation of small, long-range coupling interactions. Electronegative atoms close to C_6 such as oxygen in OTC and chlorine in CTC, shift the



(3) TC HCl

resonance significantly downfield (see Table 6). Absence of this signal narrows the range of possibilities for TC identification.

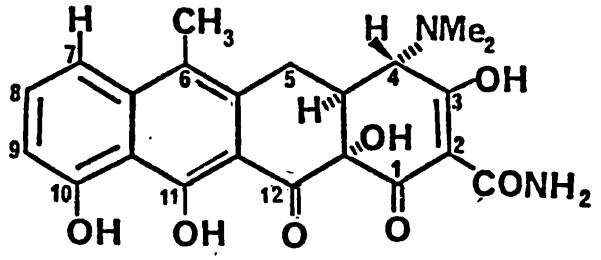
In the spectrum of anhydro TC (14), the 6-methyl proton resonance forms a sharp singlet at 2.4ppm. The downfield shift of this signal ($\delta\Delta$ 0.9ppm), is a result of the change of the C_6 carbon from sp^3 to sp^2 hybridization, and yields a significant difference between the TC(3) and anhydro TC (14) spectra. This also provides a useful means to monitor dehydration of the parent compound.

Table 6. ^{1}H-NMR chemical shift data of C_6-methyl resonance (100 MHz)

Solvent Dmso.d_6/TMS (TMS = 0 ppm)

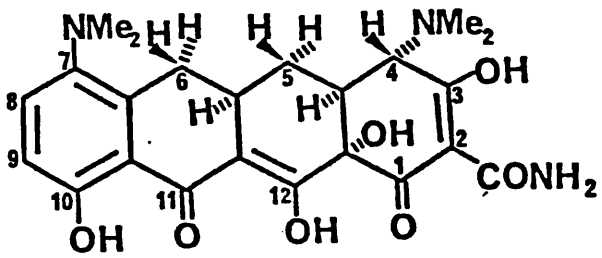
Name	C_6-methyl (ppm from TMS)	
TC HCl	1.5	broad singlet
4-epi TC HCl	1.5	broad singlet
Anhydro TC HCl	2.4	singlet
CTC HCl	1.9	singlet
6-demethyl CTC HCl	---	absent
Minocycline	---	absent
OTC HCl	1.7	broad singlet
Methacycline	---	absent
Meclocycline	---	absent
Doxycycline	1.5	broad singlet ($W_{1/2}$*=12 Hz) (doublet at 400 MHz (separation 8-9 Hz)
6-epi doxycycline	1.0	doublet at 400 MHz (separation 6Hz)

* $W_{1/2}$ is the width at half height



(14) Anhydro TC HCl

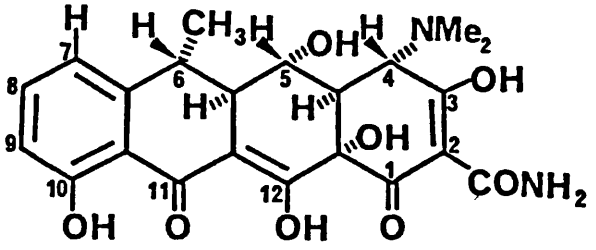
Since the stereochemical change in 4-epi TC HCl is well removed from C_6-methyl, there is no change in the chemical shift of the C_6-methyl in 4-epi TC. Absence of this resonance is of diagnostic value for minocycline (9), 6-desmethyl CTC, methacycline and meclocycline. The 6-methyl resonance of doxycycline (6) has an identical chemical shift to that of TC HCl, although a change was expected due to the loss of C_6-OH. This indicates that removal of C_6-OH is compensated by the insertion of C_5-OH. The C_6-methyl resonance appears as a very



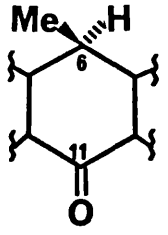
(9) minocycline HCl

broad singlet ($W_{1/2}$=12 Hz), but in the spectrum run at 400 MHz (Spectrum 6), the C_6-methyl resonance was resolved into a doublet (separation 8-9 Hz) near 1.5 ppm. The spectrum of 6-epi doxycycline (also run at 400 MHz, Spectrum 6) showed a doublet, higher field than that of the parent compound near 1.0 ppm (separation 7 Hz). This indicates that the C_6-methyl is experiencing greater deshielding in the α-configuration.

The chemical shift data for the C_6-methyl resonances are presented in Table 6.

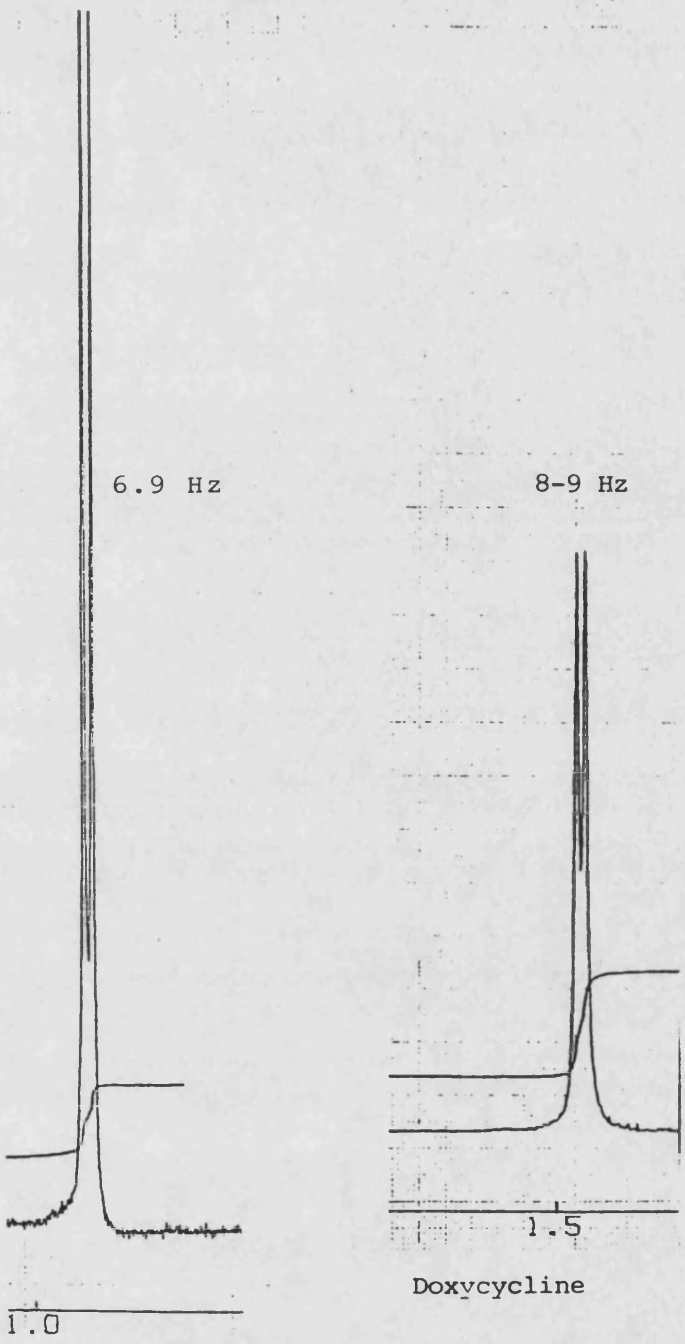


(6) Doxycycline



6-epi isomer of doxycycline

(partial structure only)



Spectrum 6. The spectrum of doxycycline and 6-epi doxycycline (in Dmso.d_6/TMS, 400 MHz) to show the shift and separation of the C_6-CH_3 resonance.

DIMETHYLAMINO PROTONS

All spectra of hydrochloride salts display intense resonances near 2.9 ppm attributable to the common C_4 quaternary substitent (H^+NMe_2). The additional and similar resonance at 2.6 ppm in the spectrum of minocycline HCl (Table 7) reveals the C_7 dimethylamino feature of this derivative. Of the two N-dimethylamino substituents, that at C_4 is the more basic and would be expected to be the preferred site of protonation of the mono-hydrochloride. This fact allows the assignment of the two resonances because N-protonation leads to a pronounced downfield shift of the N-methyl resonance (Table 7).

The broad nature of the N-dimethylamino resonance in the 220 MHz spectrum (see Spectrum 7) of OTC HCl requires comment. The 220 MHz spectrum was run in D_2O plus DCl. The broadening of the N-dimethylamino resonance of OTC HCl due to the addition of DCl was then confirmed at 100 MHz when the width at half height ($W_{1/2}$) increased from 3 to 10 Hz). Signal broadening as a result of C_4-quaternary ammonium coupling may be discounted since a large excess of D_2O is present whereby the nitrogen is deuterated rather than protonated. Therefore, the broadening is probably due to the non-equivalence of the two N-methyl environments at low pH. This situation will arise under conditions of slow proton exchange, because the two N-methyl groups are adjacent to an asymmetric centre at C_4. Any restricted rotation about the C_4-N bond should enhance the magnitude of the N-methyl chemical shift difference.

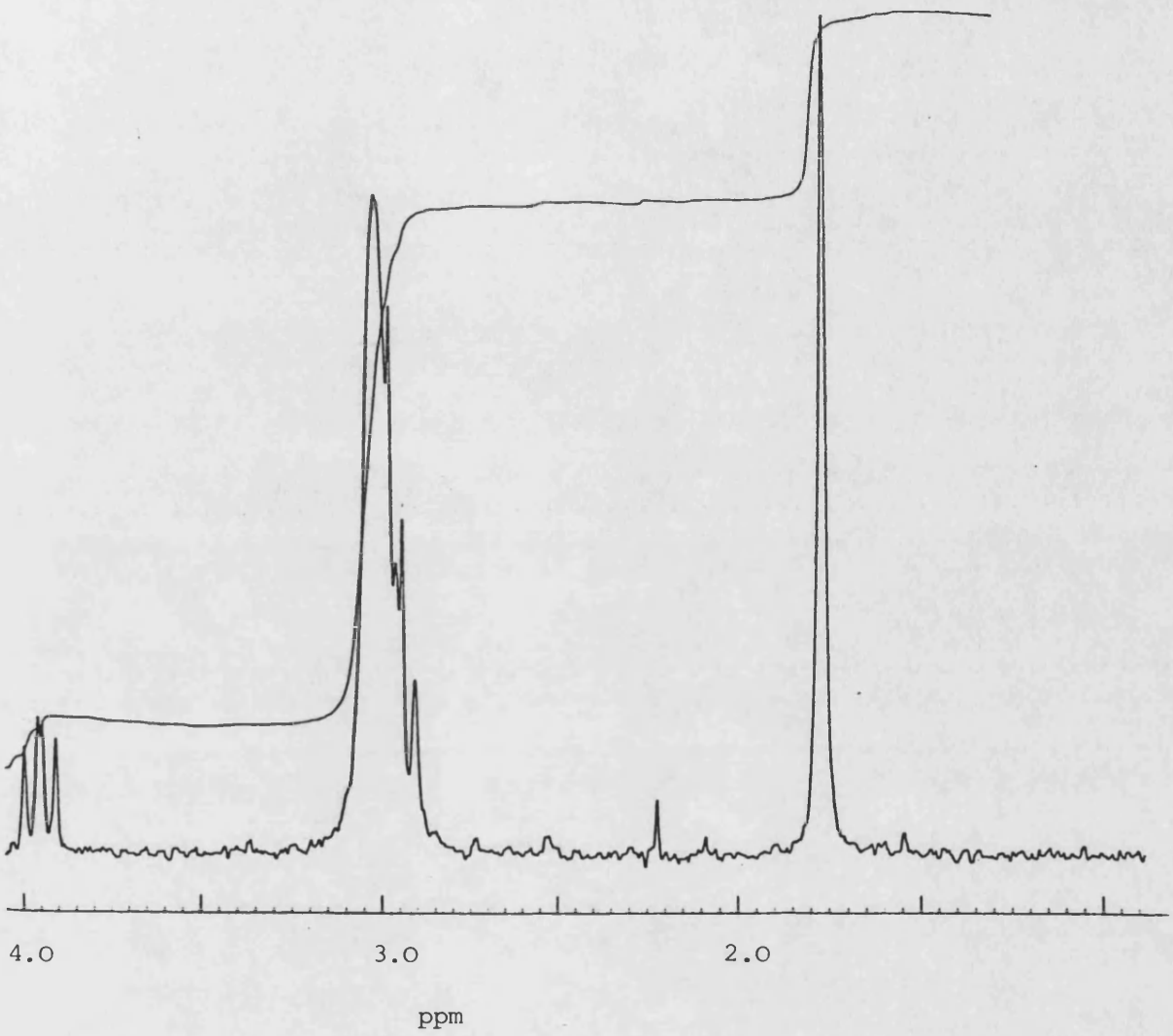
Table 7. Chemical shift data (^1H) of C_4-dimethylamino signals in Dmso.d_6/TMS. (TMS = 0 ppm)

Name	Chemical shift of C_4 (and C_7) NMe_2 substituents	$W_{1/2}$* (Hz)
TC HCL	2.9 singlet	4.6
TC Base	2.5 singlet	4.6
TC base in pyridine.d_5	2.6 singlet	3
TC HCl in pyridine.d_5	2.6 singlet	3
4-epi TC HCl	2.9, 3.0 singlets (sharp and broad respectively)	†
Anhydro TC HCl	2.9 singlet	3
CTC HCl	2.9 singlet	6
6-demethyl CTC HCl	2.9 singlet	6
Minocycline	2.9, 2.6 singlets	5.5
OTC HCl	2.9 singlet	3
OTC HCl in D_2O	3.1 singlet	5
OTC HCl in pyridine.d_5	3.1 singlet (3 Hz $\xrightarrow{DCl}$ 10 Hz)	**
Methacycline HCl	2.9 singlet	5
Doxycycline	2.9 singlet	6

* $W_{1/2}$ = width at half height at 100 MHz

** width at half height changes after adding DCl

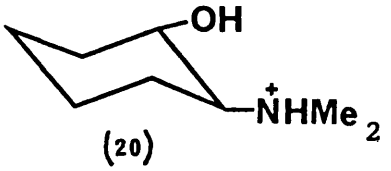
† sample contains TC HCl



Spectrum 7. 220 MHz spectrum of OTC HCl in D_2O + one drop DCl

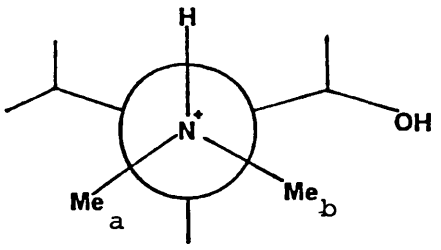
An analogous case is provided by the spectrum of the trans-2-dimethylamino-cyclohexanol hydrochloride (20), where distinct N-methyl signals are observed. This phenomenon has also been observed in the spectrum of cis-isomer, but with less pronounced separation (Wu, 1970).

NMe_2 signal:two singlets near 2.8 ppm
separation 7Hz at 60MHz in D_2O

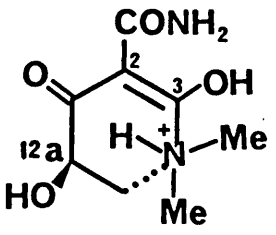


(20)

View down N-C bond of preferred conformation. Me_b is subject to influence of OH substituent whereas Me_a is not.



A similar situation may be readily envisaged for protonated TC derivatives (21). The phenominon requires a slow rate of proton (deuterium) exchange at the basic centre and hence is best observed when the pH is lowered. Separate lines should appear if the exchange rate is sufficently slow, otherwise the two resonances overlap to form a broad band, as observed in Spectrum 7 .

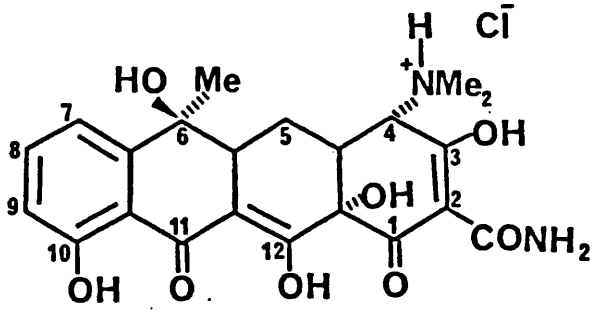


(21)

View of ring A in conformation along $N-C_4$ bond .

ACIDIC PROTONS

Tetracyclines are remarkable for their high content of exchangeable protons. Most hydrochlorides possess the eight acidic protons of TC



TC HCl (exchangeble protons)

itself (C_3-OH, C_6, C_{10}, C_{12}, C_{12a}, the amino group of the C_2-amide, the protonated C_4-quaternary ammonium function). In principle all protons are detectable by comparing the spectra in Dmso.d_6 before and after addition of D_2O. In practice, however, only four resonances attributable to the acidic protons can be assigned with certainty and then only if sufficent field offset is employed when recording the spectrum (Spectrum 4, Table 8).

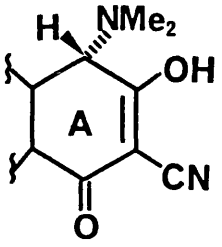
Two research groups have reported on the acidic proton resonances (chiefly those of TC itself (Asleson et al., 1974; Williamson and Everet, 1975) and a summary of the assignments is presented. A pair of broad one-proton resonances near 9 and 9.6ppm invariably appear in spectra run in Dmso.d_6. This pair is attributed to the amide protons on the basis of their absence in spectra of the corresponding 2-cyano

Table 8. Chemical shifts for the acidic protons of tetracycline group of antibiotics in Dmso.d_6/TMS. (TMS = 0 ppm)

Name	100 MHz		400 MHz	
	Acidic protons		Acidic protons	
	$CONH_2$	Others	$CONH_2$	Others
TC HCl	9.5, 9.1	11.7, 15.0	9.1, 9.55	11.8
TC base	9.1, 8.6	11.9	9.8, 10.1*	12.1
4-epi TC HCl	9.3, 9.5	11.7, 15.1		
Anhydro TC HCl	9.3, 9.7			
CTC HCl	9.0, 9.5	12.0	9.1, 9.6	12.2
6-demethyl CTC	9.1, 9.6	11.6	9.1, 9.6	12.2
Minocycline	9.0, 9.4	11.6	9.1	11.9
OTC HCl	9.1, 9.6	11.5, 14.9	9.1, 9.6	11.6
Methacycline	9.1, 9.6	6.4, 11.5, 15.0		
Doxycycline	9.1, 9.6	11.5, 15.1	9.1, 9.6	11.5, 15.2

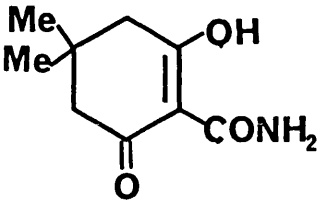
(* in pyridine.d_5)

TC analogue (22), and by reference to the spectra of model compounds such as (23) (Asleson et al. 1974) and (24).

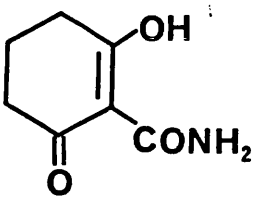


(22)

2-cyano TC



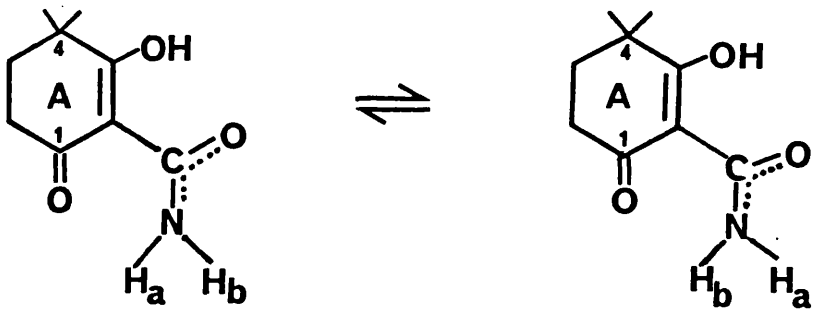
(24)



(23)

Broad resonances at 8.7 and 9.5ppm in Dmso.d_6

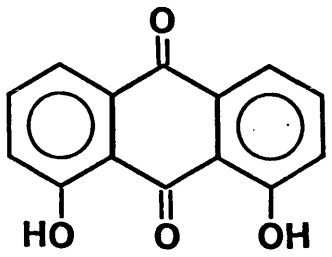
The appearance of these bands typifies protons exchanging between two different environments, such as could arise as a result of restricted rotation about the N-C amide bond (25) (Abraham & Loftus, 1980).



(25)

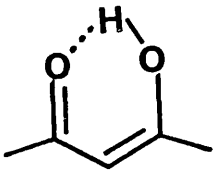
The narrower resonances at lower field near 12 and 15 ppm are assigned to the C_{10} and C_{12} hydroxyl protons, as there is evidence that these are intramolecularly hydrogen bonded to nearby carbonyl oxygen at C_{11} and C_1 respectively (Asleson et al., 1974). The deshielding consequence of hydrogen bond formation is well known especially when

intramolecular (review, Casy 1971). Data on models (26) and (27) support these assignments (Williamson and Everett, 1975).



δ OH = 12 ppm

(26)



δ OH = 15.1 ppm

(27)

The C_6 and C_{12a} hydroxyl groups probably form intermolecular hydrogen bonds with the solvent and absorb upfield of 8ppm to form less well defined bands due to overlap with other resonances. It is likely that the C_3-hydroxyl and quaternary ammonium proton absorptions are too broad for detection because of rapid exchange rates.

Acidic proton NMR data on TC derivatives are thus of little value for characterization purposes. For example, variation in acidic proton content, as in OTC with one extra and doxycycline with one less, cannot be exploited since the diagnostic signals fall above 8 ppm and cannot be resolved. When solubilities permit, D_2O is preferred to Dmso.d_6 as an NMR solvent for tetracyclines as it eliminates all separate acidic proton absorptions and makes for spectral simplicity, provided that the residual HDO band does not overlap any non-acidic proton signals.

METHINE (4H, 4a-H, 5H and 5a-H) and 5-H_2 METHYLENE SIGNALS

In this group, only the 4-H resonance is resolved in all spectra. The other signals form an unresolved envelope between 1.5 and 3.0 ppm in spectra recorded at 60 and 100 MHz, but satisfactory resolution may be achieved in most cases when the operating frequency is raised to 400 MHz (see later). Thus only one resonance, additional to the N-dimethylamino and C_6-methyl could be resolved at a field higher than 6 ppm in the spectrum of TC HCl in Dmso.d_6. This was a broad one-proton singlet at 4.3 ppm and assigned to the C_4 proton, since this should be the most deshielded of the three methine signals (it is flanked by charged nitrogen and an enolic carbon atom). The spectra of all TC samples displayed a similar band in the range 4.3-4.8 ppm (Table 9).

The 4a-H and 5a-H methine proton resonances must therefore constitute part of the unresolved envelope between 1.5 and 3 ppm. Wittenau and Blackwood (1966) reported the PMR features of TC base in pyridine and gave 3.6 ppm as the chemical shift of the 4-H proton, with a range of 2.2-3.2 ppm for the 4a-H, 5-H and 5a-H protons. They also quoted a range of 3.6-4 ppm for the 4-H proton resonance of a variety of TC derivatives, mostly bases, in pyridine and trifluoroacetic acid.
If assignment of the 4-H proton resonance of TC HCl is correct (as also supported by spin-spin decoupling experiments on TC base (Williamson and Everett, 1975), its chemical shift should be sensitive to ionisation changes at C_4-nitrogen and C_3-hydroxyl.

Table 9. Chemical shift data (60/100 MHz) for methine (4-H, 4_a-H, 5-H and 5_a-H) and 5-H_2 methylene protons. Dmso.d_6/TMS

Name	4-H	5-H	4a-H	5a-H	5-H_2
TC HCl	4.3 bs	---	a	a	a
TC base	3.3	---	a	a	a
TC basein pyridine.d_5	3.7	---	a	a	a
TC HCl in pyridine.d_5	3.7	---	a	a	a
CTC HCl	4.4 bs	---	a	a	a
6-demethylCTC	4.4 bs	---	a	a	a
	4.7 bs(C_6)				
Minocycline	4.3 bs		a	a	---
OTC HCl	4.7 bs	3.8 bt	a	a	---
OTC HCl(D_2O)	4.4 bs	4 dd	a	a	---
OTC HCl in pyridine.d_5	4.8 d	5.0 t			
Methacycline	4.7 bs		3.1 d	---	---
6-deoxy OTC	4.8 bs	a	a	a	---
AnhydroTC HCl	4.4	---	a	a	---
4-epi TC HCl*	4.75	---	a	a	---
	4.3 bs				

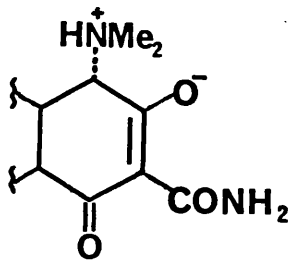
a - unresolved envelope (within 1.6-3.2 ppm); d - doublet

bs - broad singlet; * sample from Cyanamid USA, contains TC HCl

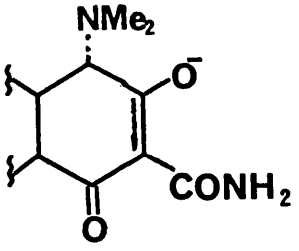
bt - broad triplet

t - triplet

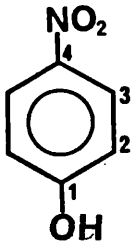
Spectra of TC base in pyridine and Dmso.d_6 provide supportive data. In these spectra the 4.3 ppm resonance of TC HCl in Dmso.d_6 shifts upfield to 3.7 ppm in pyridine, both signals forming narrow doublets (Table 9). Interpretation of these shifts is complicated by the fact that deprotonation of both the C_4-nitrogen and C_3-hydroxyl will have a shielding influence on the 4-H proton (Casy,1971). The large upfield shift seen for TC base in Dmso.d_6 in which the zwitter-ion (28a) probably predominates (Colaizzi and Klink, 1969, also page 140) suggest that shielding due to C_4-hydroxyl ionisation dominates over deshielding arising from the quaternary ammonium group. The same factor may also account for the fact that the chemical shift of the NMe_2 group of the base is at higher field (2.5 ppm) than that of HCl salt (2.9 ppm). An estimate of the magnitude of shielding consequent upon hydroxyl ionisation was gained from data on p-nitophenol (29).



(28a)



(28b)



(29)

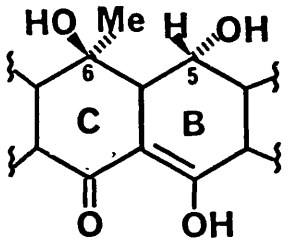
	ppm
δ2-H in Dmso.d_6	= 6.9
in Dmso.d_6/NaOH	= 6.3

The hydrochloride to base upfield shift of 4-H was less pronounced when the base was dissolved in pyridine (shift to 3.7 ppm rather than 3.3 ppm). In this case the feebly basic solvent would be expected to compete with the nitrogen of TC for the acidic protons (note that the spectra of TC HCl and TC base are identical in pyridine). For this reason a large 4-H proton shift would be anticipated because of a rise in the population of the species (28b), where both ionisable functions are in a favourable state for shielding the 4-H proton. However, when pyridine complexes with polar molecules, protons in the vicinity of association sites are often markedly deshielded, because they fall within the aryl deshielding region of the solvent molecule as a result of the geometry of the solute-solvent association mode (Demarco et al., 1968; Wenkert and Mylari, 1967). It is probable that the same deshielding mechanism operates in the case of TC-pyridine, where association may be through the ion-pair formation of the type $O^{-}....HN^{+}$, to shift the C_4-H to lower field than its position in Dmso.d_6. Low field shifts of C_4-NMe_2, C_6-methyl and C_3-amide were also apparent.

The narrow profile of the 4-H proton signal suggest a tetracycline structure in which the ring **A** is bent approximately at 90° to the plane of rings **B**, **C** and **D** (page 72). There is evidence that this is the preferred solute conformation of most of the tetracycline derivatives (see later), as established for CTC and some of its analogues in the solid state by X-ray diffraction (Donohue, 1963). In (Fig.3 page 72) the dihedral angle relating the C_4 and C_{4a} protons is close to

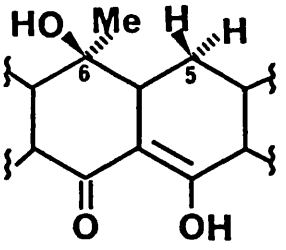
60^{o}, while in the conformation in which all the rings are planar the same angle is close to 180^{o}. Such magnitudes are associated with small and large vicinal couplings respectively (review Casy, 1971).

In 5-oxytetracycline derivatives (i.e. OTC, doxycycline , methacycline and meclocycline), the 5-H resonance is also resolved as is 6-H proton in 6-demethyl CTC as a result of deshielding by the geminal oxygen (30a, 30b).



OTC derivatives

(30a)



TC derivatives

(30b)

The spectra of most TC HCl samples displayed a low intensity broad band or narrow doublet to low field of the 4-H proton resonance, which is indicative of the presence of 4-epi TC HCl as an impurity i.e. a broad band near 4.8 ppm in Dmso.d_6 and a doublet (line separation 3 Hz) at 4.6 ppm in pyridine.d_5. A reputed sample of 4-epi TC HCl, supplied by Cyanamid, USA., also displayed duplicate 4-H proton resonances in its spectrum (Table 9). These signals provide the best means of assessing isomeric purity by ^{1}H NMR, since signal duplication in the N-dimethyl amino absorption region is complex.

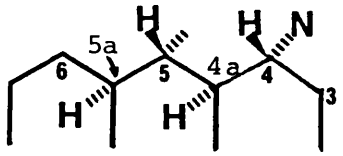
A similar situation was observed with CTC HCl and its 4-epi isomer. The CTC HCl sample showed a broad singlet at 4.3 ppm assigned to 4-H, while the 4-epi CTC HCl (Pfizer sample) showed a doublet lower field to that of the parent drug at 4.7 ppm (separation 3.8 Hz). The isomer impurity was confirmed by HPLC where the main peak was preceeded by a low intensity peak.

6-demethyl CTC HCl displayed two single-proton singlets near 4.5 ppm in its spectrum. The higher field resonance at 4.4 ppm assigned to 4-H as usual, while the signal at 4.7 ppm must arise from the 6-H proton which is deshielded by its oxygen and chlorine neighbours. The low order of coupling ($^3J_{5a,6}$) evident from the profile of the 6-H signal indicates that the 6-hydroxyl of this compound is pseudo-axial in the half chair conformation (ring B) of the molecule in Dmso.d_6, supplementing the rather sparse evidence of stereochemistry of this derivative (Mitscher, 1978). Higher resolution data supports this conclusion (page 82)

HIGH RESOLUTION SPECTRA RECORDED ABOVE 200 MHz

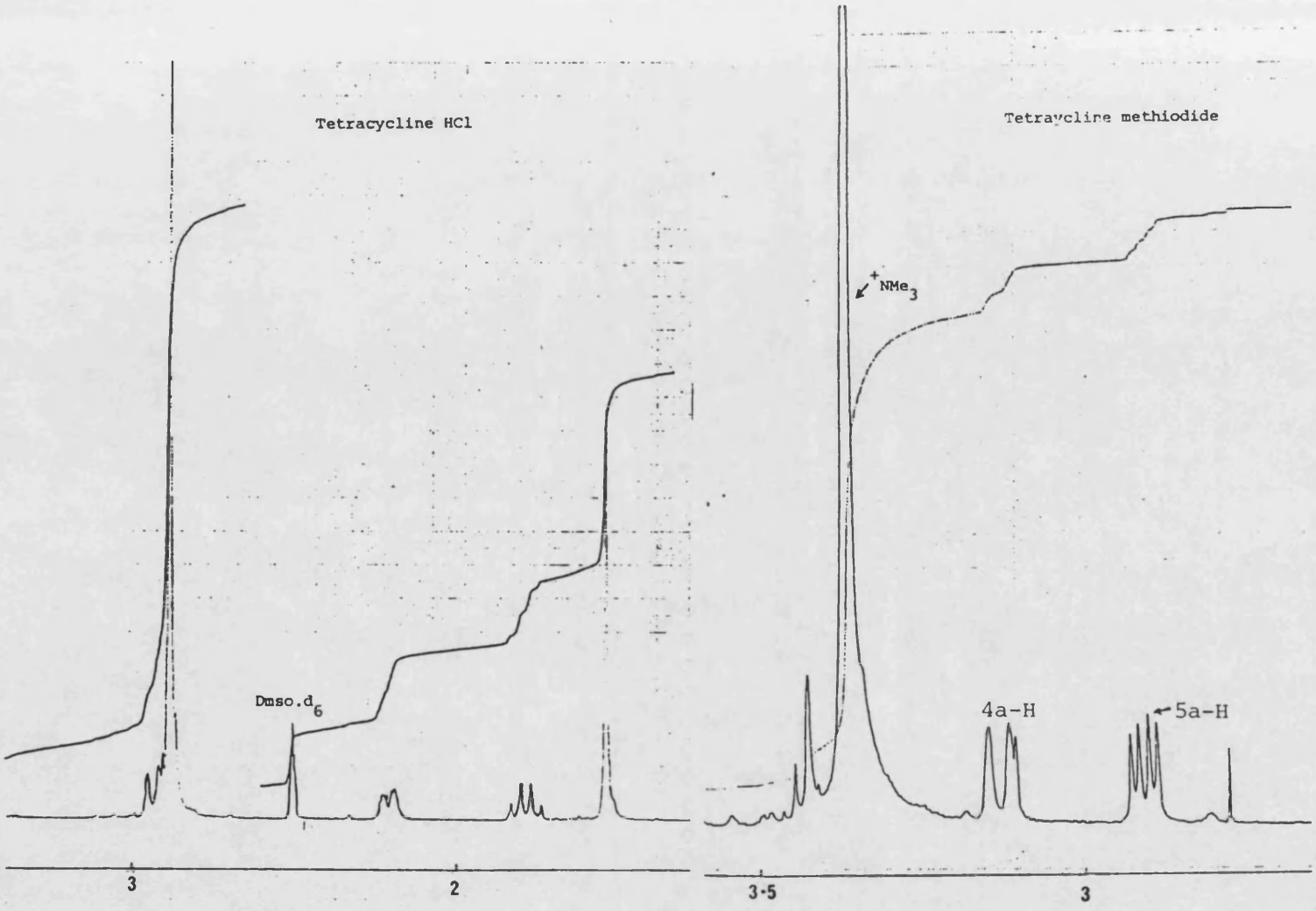
Methine and methylene proton resonances were generally well resolved in spectra recorded at 400 MHz, except for 4a-H and 5a-H signals which were partially masked by that of the N-dimethyl amino resonance. However, in the spectrum of TC methiodide, the $^{+}NMe_3$ signal was displayed to lower field than that of $^{+}NMe_2$ in the HCl spectrum, revealing well resolved 4a-H and 5a-H protons (see Spectrum 8) The 400 MHz chemical shift data are recorded in Table 10.

The assignments were mostly trivial and coupling interactions were substantiated in some cases by spin decoupling experiments. Of the two closely placed 4a-H and 5a-H multiplets near 3 ppm, that to lower field could be assigned to 5a-H in most spectra, since it did not include the small coupling displayed by the 4-H signal (31).



(31)

The 5-H methylene signal near 1.7 ppm, which displayed three large couplings (with 4a-H, 5a-H and a geminal hydrogen rsulting in an apparent quartet) was assigned to the pseudoaxial- β -proton, and



Spectrum 8. 400 MHz spectrum of tetracycline HCl and tetracycline methiodide in Dmso.d_6/TMS

Table 10.

Compound	C_6-Me (+ 6-H)	C_4-NMe_2	4-H	4a-H	5-H	5a-H	C_6-H
TC HCl	1.54 s	2.89 bs	4.23 d (.84) (4.33 bs at 25°C)	2.87 dd (.84)	α:2.18 ddd (2.8, 5, 13.6) β:1.79 bq (11-13)	2.91 dd (5.2, 10.8)	------
CTC HCl	1.84 s	2.87 bs	4.32 bs	2.92 bd (13)	α:2.24 ddd (2, 5, 13) β:1.71 bq (10.5)	2.99 dd (5.2, 10.5)	------
4-epi CTC HCl	1.80 s	broad band beneath 4a-H and 5a-H	4.74 d (3.80)	2.91 dt (4, 4, 14)	α:2.17 bq (14) β:1.58 bq (10.5)	3.0 dd (5, 11)	------
Doxycycline ethanol signals at 6.5, 13 ppm	1.45 d (6.6) 6-H at 2.64 ppm (m)	2.80 bs	4.7 s	2.88 d (11.3)	β:3.48 bt (8-10)	2.55 dd (8.2, 12.4)	------
6-epi doxycycline	0.9 d (6.9) 6-H at 3.21 m (4, 6.9)	2.78 bs	4.6 s	2.93 d (11.6)	β:3.38 (7, 11)	3.0 (4.2, 9.5)	------
Minocycline	------	2.95 bs C_7-NMe_2 at 2.6 ppm bs	4.4 bs	2.3 bd	α:3 bs	------	α:2.5 β:3.3 bd
Meclocycline	C_6 is methylene at 5.6 (two broad singlets)	2.8 bs	4.5 bs	3.55 bd (8.97)	β:3.8 bt	3.0 (11.36)	------
TC methyl-iodide	1.51 s	3.37 bs (NMe_3 signal)	4.42 bs	3.13 bs	α:2.25 bd (13) β:1.74 bq (13)	2.91 dd (5.1, 11.2)	------
β-apo OTC base	2.38 s	1.80	3.5 d (12)	2.73 bt (0.8, 11.5)	6.06 d (0.8)	4.3 d (11.5)	------
α-apo OTC base	2.4 s	2.05	3.18 d (5.7)	3.06 bt (5-6)	5.85 s	4.6 d	------

^{1}H NMR chemical shifts (in ppm from TMS at 0 ppm) and coupling constants (in parentheses following chemical shifts) of tetracycline antibiotics and their common degradation products measured at 400 MHz in Dmso.d_6/ TMS.

b, broad , s, singlet , d, doublet , t, triplet , q, quartet , m, multiplet

that near 2.2 ppm (showing one large coupling with the geminal proton, and two small couplings with 4a-H and 5a-H) was assigned to the pseudoequatorial- α -proton (see Fig 3 , and Spectrum 8)

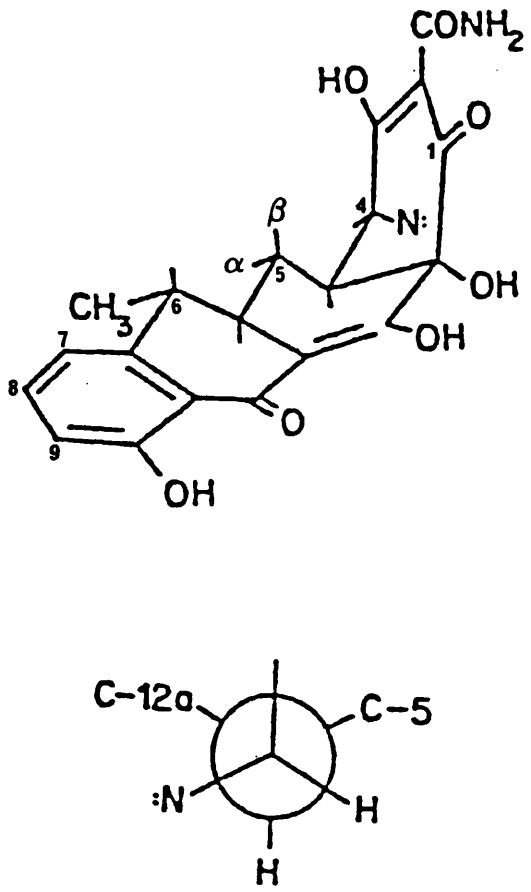


Fig. 3 Conformation of tetracycline hydrochloride derivatives. Newman diagram depicts view down the C_4-C_{4a} bond.

Magnitudes of the coupling constants for protons attached to C_5, C_{5a}, C_4 and C_{4a} provide evidence for the geometry of rings **A** and **B** of the tetracycline derivatives as hydrochloride salts. The results are in general agreement with conformations of the type established for TC HCl, CTC HCl, OTC HCl and 6-demethyl CTC HCl in the solid state (Donohue et al., 1963; Dunitz and Robertson, 1952). In this arrangement (Fig 3) ring **A** is a half chair aligned approximately at right angles to the mean plane of rings B-D.

NMR data for 4-epi CTC require a conformation with a small dihedral angle linking 4-H and 4a-H ($^3J_{4,4a}$ 3.8 Hz) and one in which the dimethylamino group is in a highly hindered environment to allow for its very broad resonance (Fig 4). A conformation such as that in Fig. 3 is possible (with 4-H and 4-NMe_2 reversed, but the one depicted in Fig 4, where the NMe_2 group is markedly hindered and H-4-C-4-C-4a-H-4a dihedral angle is probably different from that in Fig 3 (leading to $^3J_{4,4a}$ coupling difference magnitudes for the epimeric pair) is more likely. Conformations of the other derivatives with supportive coupling constant evidence will be presented later.

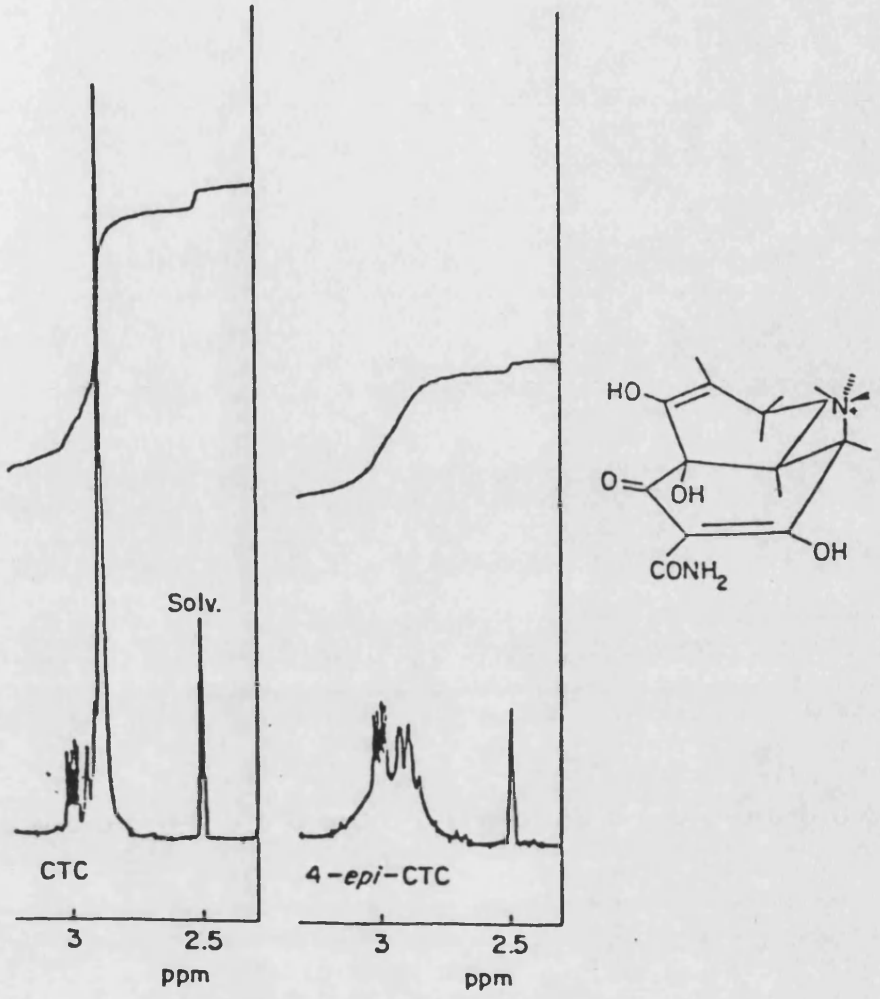
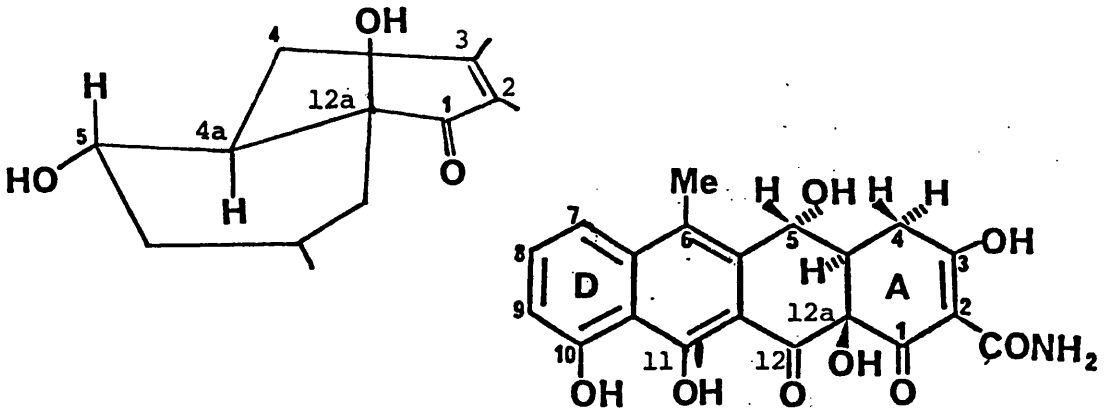


Fig. 4. 3ppm region of the 400 MHz ^{1}H NMR spectra of CTC and 4-epi CTC in Dmso.d_6 showing NMe_2, 4a-H and 5a-H signals. A conformation of 4-epi CTC (partial structure) which places the dimethylamino group in a highly hindered environment is shown alongside.

EVIDENCE OF CONFIGURATION

OTC HCl

Chemical evidence for the configuration of OTC was presented by Wittenau et al. (1965). Since OTC may be able to assume a variety of conformations, 12a-epi-dedimethylaminoanhydro-OTC (32) was used, which can exist in only one conformation, determined by the trans junction relationship of the rings A-B. With subsequent NMR analysis, the conformation of protons at C_5 and C_{4a} were thus shown to be trans diaxial (according to the ^3J values), and the hydroxy group at C_5 to be cis to the hydrogen atom at C_{4a}.



(32) 12a-epi dedimethylamino anhydro OTC

In the spectrum of OTC HCl run at 100 MHz, the broad singlet at 4.7 ppm was assigned to 4-H, and the one at 3.8 (a broad triplet separation 8Hz) was assigned to 5-H. A subsequent run at 220 MHz, resolved the broad triplet into a doublet of doublets (with separations of 8.8 and 11 Hz). Hence, the main feature of the 5-H resonance i.e. two large couplings indicate its configuration as pseudoaxial (β)

to 4a and 5a protons. Therefore 5-OH is pseudo-equatorial (α) in configuration as shown in Fig. 5.

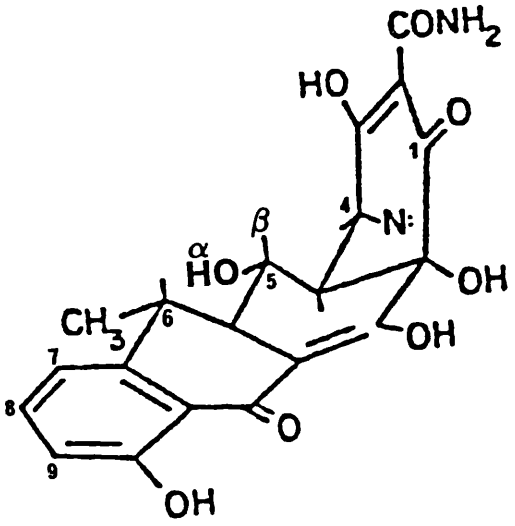
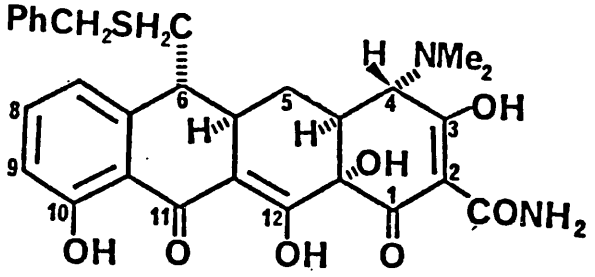


Fig. 5. The stereochemical configuration of OTC HCl

DOXYCYCLINE AND 6-EPI DOXYCYCLINE

Chemical evidence for the configuration of doxycycline was presented by Stephen et al (1963). They found that hydrogenation of methacycline yielded an approximately equimolar, readily separable mixture of the two C_6 epimers of doxycycline. Evidence of the C_6 stereochemistry was obtained by a stereo-specific synthesis of doxycycline. It was observed that benzyl mercaptan will add to the methylene group of methacycline through a free radical mechanism to give (33).



(33)

Only one C_6 epimer is obtained. From a model study, it was concluded that the more stable form is the one with the larger group at C_6 equatorial (or α) i.e. similar to that of 6-Me in OTC as shown in Fig. 5. , This conclusion is now confirmed from the chemical shift data presented in Table 10 . The 4a-H resonance (at 2.88 ppm) is a doublet with a large coupling to 5-H (11.3 Hz). The 5-H resonance, at 3.48 ppm, is a broad triplet with large couplings to both 4a-H and 5a-H (8-10 Hz). These coupling constant magnitudes show that 5-H must be β (i.e. axial) and 5-OH α (equatorial).

The 5a-H resonance (at 2.55 ppm) is a doublet of doublets showing two large coupling constants of 8.2 and 12.4 Hz (due to coupling with 5-H and 6-H), therefore 6-H must be β , thus proving C_6-methyl to be α in configuration. Therefore, the coupling constants are consistent with the chemical evidence of configuration, as presented in Fig 6.

In the 6-epi doxycycline isomer, the position of C_6-methyl and C_6-H are reversed. If this stereochemistry is correct the following

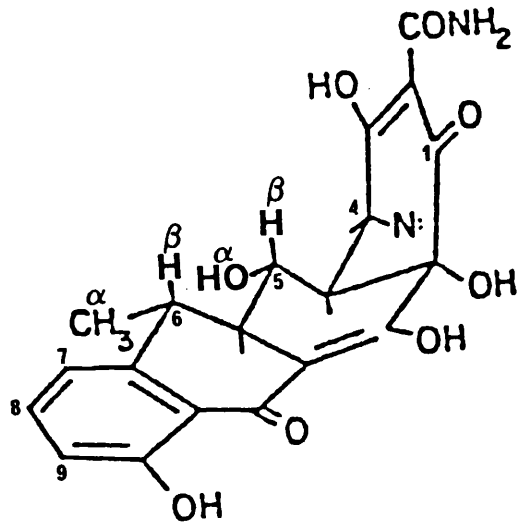


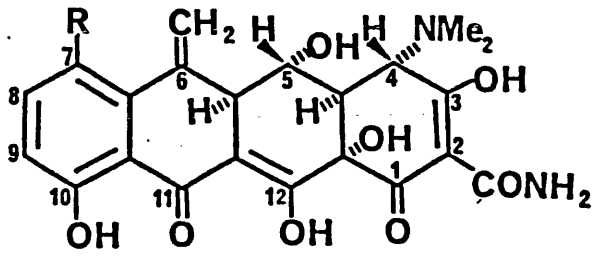
Fig. 6 Conformation of 6-deoxy OTC

changes in coupling constants should be observed:

	Doxycycline	6-epi doxycycline
C_{4a}-H	doublet. large coupling (11.3 Hz)	no real change expected (11.6 Hz)
C_5-H	broad triplet. Two large couplings (8-10 Hz)	no real change expected (7,11 Hz)
C_{5a}-H	doublet of doublets Two large couplings (8.2, 12.4 Hz)	should show one large coupling with C_5-H and one small coupling with C_6-H (4.2 and 9.5 Hz)

The coupling constants are witness to the fact that in the 6-epi isomer, the C_6-methyl is β (pseudo-axial) and therefore C_6-H must be α (pseudo-equatorial).

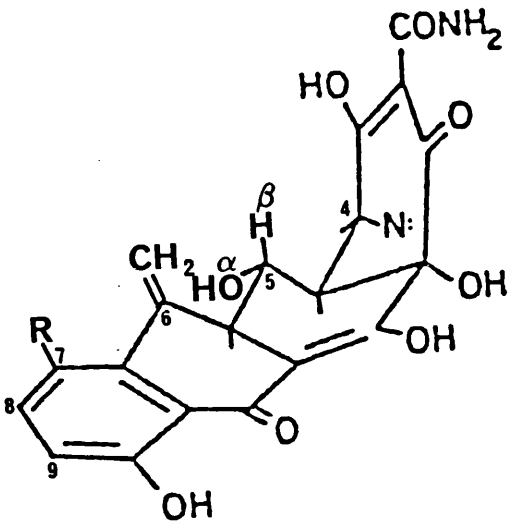
MECLOCYCLINE (R=Cl) AND METHACYCLINE (R=H)



(34) Meclocycline and methacycline

Interpretation of the ^1H NMR spectral features of meclocycline and methacycline in terms of configuration is fairly straighforward and supports a conformation similar to that of OTC HCl. The 4-H resonance is a broad singlet at 4.5 ppm. Between 3 and 4 ppm, there are three resonances, two doublets and one triplet. These must be due to C_{4a}, C_{5a} (two doublets with large couplings of 9.0 and 11.4 Hz respectively), and C_5-H (a triplet with two large couplings with C_{4a}-H and C_{5a}-H). Therefore, C_5-H has β (pseudo-axial) geometry.

Two broad singlets at 5.6 ppm must be due to the C_6-methylene group. The slight broadness of each signal is due to small geminal couplings (typical of a $=CH_2$ system). The conformation at C_4 is as that of OTC HCl i.e. C_4-H is α (pseudo-equatorial). The conformation of meclocycline and methacyclune is shown in Fig 7.



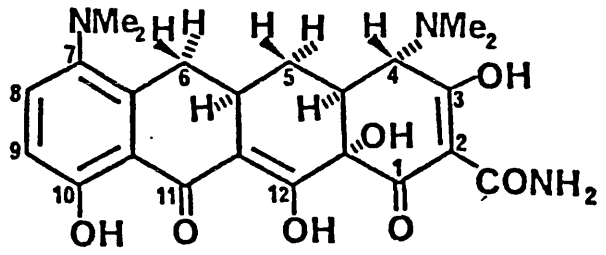
R = Cl in meclocycline
R = H in methacycline

Fig. 7 Configuration of methacycline and meclocycline

MINOCYCLINE HYDROCHLORIDE

Full resolution of ring B, C and D protons (9) was not possible at 270 MHz because of their extensively coupled nature. However, the 4-H resonance formed an isolated resonance as usual, namely a broad singlet at 4.4 ppm, typical of tetracycline HCl spectra. A lower field resonance of weak intensity at 4.8 ppm is probably due to the 4-epi isomer as an impurity, a conclusion supported by the duplication of the aromatic signals and the higher field NMe_2 signals in the same intensity sense. A sample of minocycline run by HPLC failed to show any contamination by the 4-epi isomer. This may be due to the fact that minocycline plus the isomer are eluting almost

instantaneously as in the case of TC and 4-epi TC HCl.



(9) minocycline

In a spectrum of the antibiotic , in Dmso.d_6, run at 400 MHz the following signals were resolved:

1) 4.3 ppm broad singlet (as usual) assigned to 4-H.

2) 3.1 ppm well defined doublet of doublets, separations 4.0 and 15.0 Hz. The magnitude of the larger coupling rules out a vicinal interaction (these rarely exceed 12 Hz) and it is more likely due to a geminal proton. The signal may therefore be assigned to the 6 α-H proton which interacts geminally with 6 β-H and vicinally (small coupling) with 5a-H.

3) Overlapping multiplets near 2.9 ppm, comprising the 4a-H and 5a-H signals (see below).

4) overlapping multiplets near 2.2 ppm comprising the 5 α-H and 6 β-H signals.

5) Broad (apparent quartet) near 1.5 ppm (separation 13 Hz) assigned to the 5 β-H proton.

2D correlations (COSY) experiments confirmed these assignments since they established coupling interactions between:

4.2 and 2.85 ppm resonances (4-H/4a-H)

3.2 (6α-H) with both 2.85 (5a-H) and 2.2 (6β-H)

2.2 ppm (5α-H) and 1.5 ppm (5β-H)

2.85 ppm (4α-H) and both 2.2 ppm (5α-H) and 1.5 ppm (5β-H)

All the coupling data, especially that relating to 4-H and 5a-H, points to minocycline HCl favouring a solute conformation similar to that of TC HCl.

6-DEMETHYL CTC HCl

The 400 MHz spectrum of this derivative, in CD_3OD, revealed the 4-H and 6-H resonances as clear narrow doublets:

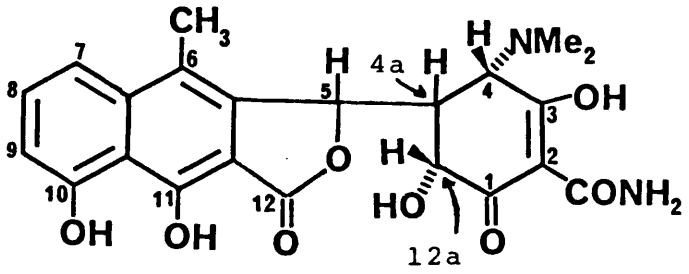
4-H 4.18 ppm separation 2.5 Hz

6-H 5.0 ppm separation 2.7 Hz

The 5a-H signal was also resolved, a multiplet near 3.2 ppm of separation 2.7, 6.8 and 9.5 Hz. All these data support a pseudoaxial (β) orientation for the 6-hydroxyl group and an overall conformation similar to that of TC HCl.

α AND β -APO OXYTETRACYCLINE BASES

Hochstein et al. (1953) reported the synthetic route leading to the formation of α and β -apo OTC (formulated as the lactones (16) of unknown stereochemistry) from OTC under acidic conditions. The reaction involves the loss of water between C_6 and C_{5a}, inversion of C_5 and rearrangement.



(16) apo-OTC base

The ^{13}C NMR spectra is consistent with the lactone formulation (see later), but the exact difference between the isomers was not revealed. To seek evidence of configuration the 400 MHz ^{1}H spectra of the isomeric pair were examined to establish the magnitude of coupling interactions within the system 5-H, 4a-H, 4-H and 12a-H. Spectra were best resolved in Dmso.d_6 at 70°C

Spectral details and assignments are summarised below:

The α and β 6-Me chemical shifts are close to that of 6-Me in anhydro TC in support of ring B being fully aromatic. Four

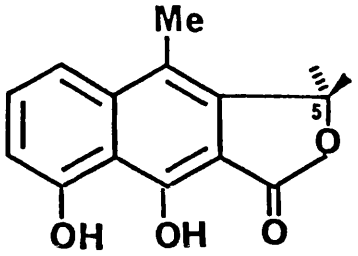
methine signals were located in the β -spectrum:

1. 6.06 ppm very narrow doublet (separation 0.8 Hz)
2. 4.3 ppm clear doublet, large separation (12 Hz)
3. 3.5 ppm doublet of large separation (12 Hz), but obscured by HDO band. The doublet was clearly resolved after addition of D_2O
4. 2.73 ppm triplet (separation 11.5 Hz), each line of which shows a small separation (0.8Hz)

Spin-decoupling experiments established that signals 2, 3 and 4 were coupled. Signal 4 was coupled in equal degree (11.5 Hz) to signals 2 and 3, and weakly (0.8 Hz) to signal 1, and must therefore arise from 4a-H. The narrow doublet near 6 ppm may be assigned to 5-H on chemical shift grounds (this proton is flanked by oxygen and an aromatic carbon). Likewise the 4.3 ppm doublet is attributed to 12a-H (vicinal to oxygen) and the 3.5 ppm doublet to 4-H (vicinal to nitrogen). Similar arguments were used to assign the α -spectrum; 4a-H was similar in chemical shift and multiplicities to that of β -4a-H, but couplings to 4-H and 12a-H were weaker (^3J 5.7 and 5.1 Hz respectively).

In the β -isomer, and to a lesser extent in the α -isomer, the dimethylamino resonance was much higher field (β 1.8 ppm; α 2.05 ppm) than the normal range of 2.5-3.0 ppm. This fact, together with the β -methine coupling magnitudes, is now applied to the configurational assignment of the lactone (16).

Since the phthalide unit (35) is in one plane, the configuration at C_5 is not a problem, but the cyclohexenone unit (36) can adopt two interconvertable half chair conformations. In the case of the β-isomer the conformation which allows the strong coupling of 4a-H to both 4-H and 12a-H) should represent the preferred conformation. Since the coupling between 5-H and 4a-H is small (0.8 Hz), the relevant dihedral angle is most probably in the range 60-90°. A β-modelincorporatingone of the two possible arrangements that provide this angle, together with conformation (37) (which provide for large couplings between 4a-H and 4-H/12a-H) places the dimethylamino group beneath, and thus shielded by the aromatic rings C-D which accounts for the unusually high field chemical shift of these protons. A similar arrangement can be derived from the mirror image of structure 37 and the two models are of the same relative configuration. The β-stereochemistry thus deduced (38) is related in configuration to the precursor molecule (i.e. OTC) at C_4, C_{4a} and C_{12a}. Since only the bond between C_{12} and C_{12a} is cleaved, it is most probable that site of epimerization is C_{12a}. Therefore the α-isomer must have the configuration 38 with the substituents at C_{12a} interchanged. The 400 MHz (^{1}H) data for these particular protons are listed in Table 10.



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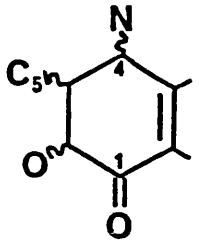

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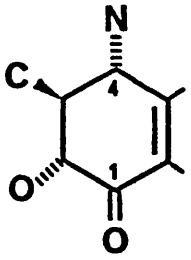

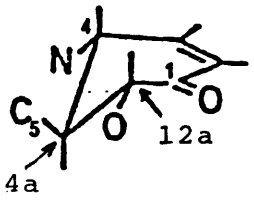

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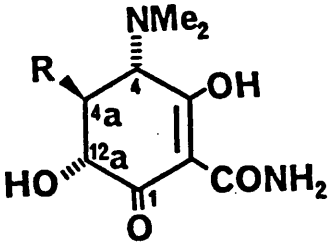


(38) (R = phthalide unit 35)

CHAPTER 3

^{13}C NUCLEAR MAGNETIC RESONANCE

Section 1 Nuclear relaxation

Introduction to ^{13}C relaxation mechanisms

A) Spin-lattice relaxation (T_1)

B) Spin-spin relaxation

Spin-lattice relaxation

Dipole-dipole relaxation

Measurement of the NOE factor

Experimental

Conclusions

Measurement of T_1 relaxation times

Introduction

Results and discussion

Justification of assignments (TC HCl)

Quaternary carbons (Cq)

Low field resonances

Protonated carbons

Oxytetracycline HCl

Doxycycline

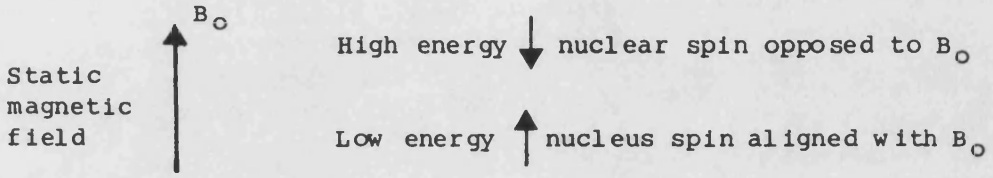
NUCLEAR RELAXATION

When a sample is introduced into a magnetic field, the nuclear spins distribute themselves, at ordinary temperature, between the low and higher energy states, with the number of spins in the low energy state being slightly higher than the latter state according to the Boltzmann distribution law. When an rf pulse is applied, the nuclear spins are perturbed and begin to relax back to the original position as soon as the rf pulse is stopped. This gives rise to the concept of nuclear relaxation. The relaxation times of carbons are very dependant on the proton environment (as discussed on following pages), and this concept has been utilised in the assignment of the various carbons of the tetracycline molecule. It is for this reason that this chapter is included at this point. The emphasis, in this chapter, will be placed on the basic theory of nuclear relaxation, various experimental designs and a very brief discussion of the assignments.

^{13}C RELAXATION TIMES

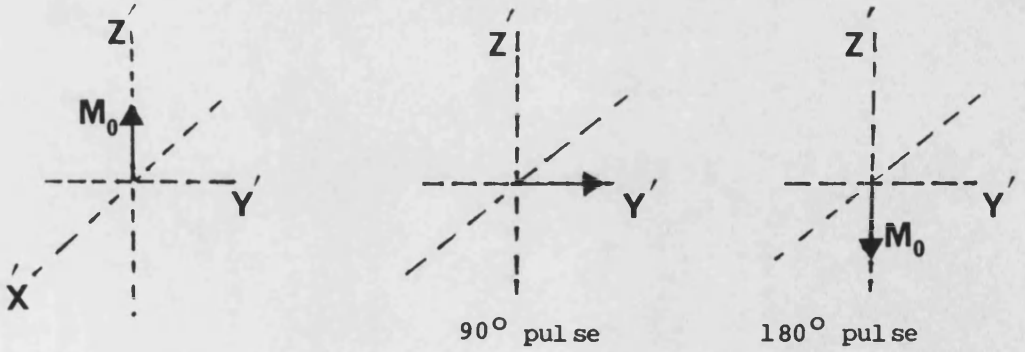
INTRODUCTION TO ^{13}C RELAXATION MECHANISMS

In a static magnetic field, B_o, an isolated nucleus of spin 1/2 has two available energy states.



According to the Boltzmann distribution law, at ordinary temperatures, only a small excess of nuclei is in the lower energy state which gives rise to an observable NMR signal.

When a sample is introducted into the magnetic field B_o, interactions between the ^{13}C nuclei and the lattice result in establishment of an equilibrium excess, giving rise to a net magnetisation vector (M_o), aligned with B_o.



M_o= stationary bulk magnetisation vector
(as represented by the rotating frame)

When the sample is irradiated with a rf pulse, the magnetic moment of

the rf turns M_o out of alignment with B_o (z axis) and towards the y axis. The duration of the rf pulse determines the angle M_o is deflected from the z axis. As soon as the rf pulse is stopped, the magnetisation vector begins to relax back towards the equilibrium position. This is achieved by two processes, spin-lattice and spin-spin relaxation.

A) SPIN-LATTICE RELAXATION (T_1)

The magnetisation vector remaining along the z-axis relaxes back along the z-axis to its original value of M_o by means of an exponential decay characterised by a relaxation time T_1. This relaxation results in loss of energy from the excited nuclear spins to the surrounding molecular lattice (see Fig. 8).

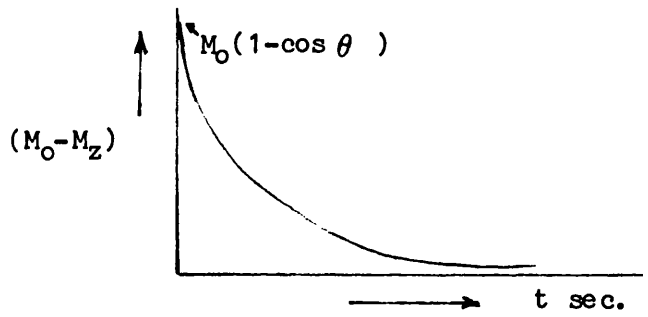


Fig. 8 Relaxation along the z-axis (spin-lattice relaxation)

This can be expressed mathematically as :-

$$(M_o-M_z) = M_o(1-\cos\theta)\exp(-t/T_1) \quad \ldots\ldots\ldots(1)$$

The spectrometers only detect signals along the Y'-axis and in the absence of T_2 (see later) the signal will decay according to

$$M_{y'(t)} = M_{y'(o)} \exp(-t/T_1) \qquad \text{.....(2)}$$

Hence, after a time $5xT_1$, it will have decayed to 0.007 $M_{y'(o)}$ i.e. essentailly zero.

B) SPIN-SPIN RELAXATION (T_2)

The second process is known as spin-spin relaxation because the nuclear spins interchange energy with one another. It also gives rise to an exponential decay.

SPIN-LATTICE RELAXATION

The process involves energy exchange between individual nuclear spins and the surrounding liquid or solid lattice. Therefore the lattice acts as a "heat sink" to establish and restore thermal equilibrium. There are a number of mechanisms which can contribute to spin-lattice relaxation in a molecule. The most common of these, along with their relaxation times are:

1) Dipole-dipole relaxation ($T_{1\ DD}$)
   a) with ^1H nucleus
   b) with other nuclei
   c) with unpaired spins e.g. dissolved oxygen

2) Spin-rotation relaxation ($T_{1\ SR}$)

3) Chemical shift anisotropy ($T_{1\ CSA}$)

4) Scalar relaxation ($T_{1\ SC}$)

Each of these contribute to produce an overall spin-lattice relaxation time T_1

$$1/T_1 = 1/T_{1\ DD} + 1/T_{1\ SR} + 1/T_{1\ CSA} + 1/T_{1\ SC}$$

The main mechanism of relaxation in most cases is via the dipole-dipole interaction (Abraham and Loftus, 1980) although other mechanisms contribute , therefore it is described in more detail.

DIPOLE-DIPOLE RELAXATION

When the nucleus undergoing relaxation is directly bonded to a second nucleus possessing a magnetic spin, then there is the possibilty of an efficent relaxation mechanism. If the ^{13}C nucleus is bonded directly to a ^{1}H nucleus, the two spins can be considered as small dipoles located at the centre of the ^{13}C and ^{1}H atoms. The ^{13}C nucleus would experience a small field due to the dipolar interaction with the proton, depending on the magnitude of the two dipoles (μ_C and μ_H respectively) and the orientation (θ) of their line of interaction relative to the magnetic field of the spectrometer (see Fig. 9)

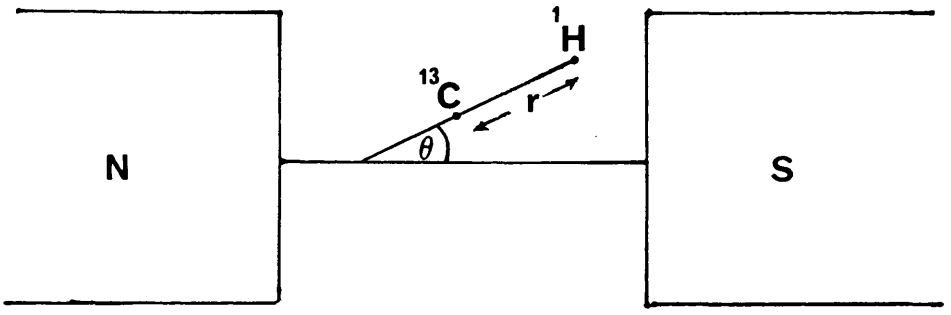


Fig. 9 Orientation of the dipolar interaction relative to the magnetic field.

The magnetic field, H_{DD}, created at the ^{13}C nucleus is given by:-

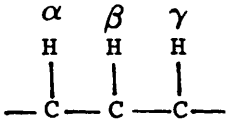
$$H_{DD} = \frac{\gamma_H \, h}{4 \, \pi \, r^3} \quad (3 \cos^2 \theta - 1) \qquad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

γ_H = magnetogyric ratio of H.
h = Planks constant.
r = distance between C and H.

As the molecule is spinning in the sample the variations in θ will cause fluctuations in H_{DD}. Relaxation can be induced by any oscillating electric or magnetic field which has a component at or close to the Larmor frequency of the nucleus concerned. Consequently the oscillation in H_{DD} constitutes a relaxation mechanism.

Wehrli (1974), has reported on the potential of T_1 measurements as an

aid to the assignemnt to the ^{13}C NMR spectra. A mathematical formula was devised to show that the individual T_1 values are related to the distance of the carbon nucleus from the nearby protons, provided the ^{13}C nuclei of the molecule relaxed predominantly by the dipolar mechanism. The relaxation rate was reported to be inversely proportional to r^6 where r is the distance between C and H atoms. The dipole-dipole contribution to relaxation falls off rapidly with distance. Protonated carbons are most effectively relaxed in this manner ($1/T_{1\ DD} \propto$ no. of directly bound protons), but in their absence, as in quaternary carbons, the presence of β and γ hydrogens will influence the $T_{1\ DD}$ magnitude



Hence, the magnitude of the $T_{1\ DD}$ values of the quaternary carbons may be related to the count of nearby β and γ protons and thereby aid assignments.

Since spectra of TC and its derivatives present the problem of assigning numerous quaternary carbon resonances, it is clear that a study of the relative $T_{1\ DD}$ values of such molecules should be of value in ^{13}C spectral assignments. A brief account of the measurements and application of T_1 values to TC HCl was described by Asleson (1975). The author used Dmso.d_6 as the solvent and therefore

had to estimate the T_1 values of carbon atoms obscured by the solvent multiplet. In the present work this problem was eliminated by the use of water as the solvent in certain cases.

Before carrying out T_1 measurements, it was necessary to obtain evidence in support of the fact that relaxation was mainly by the dipole-dipole interaction. If this is the case, the NOE factor should approach its maximum value of 2.9 since this factor depends entirely on such mechanisms.

$1/T_{1\ DD} = 1/T_{1\ obs} \times \eta / \eta_o$ $\quad \eta$ = observed NOE factor

η_o = maximum value (2.9)

when $\eta = \eta_o$ then $1/T_{1\ DD} = 1/T_{1\ obs}$

MEASUREMENT OF THE NOE FACTOR (η)

In principle, all the individual ^{13}C NOE enhancements can be determined simultaneously by comparing the intensities of a complete decoupled (no multiplets, NOE operates) and the corresponding undecoupled (multiplets, no NOE operates) spectrum (Martin and Martin, 1980). But this leads to error in calculation due to overlapping multiplets and base line adjustments. Fortunately it is possible to obtain a spectrum, using gated decoupling mode, which is devoid of the NOE effects yet is free from multiplets (Freeman et al. 1972)

The period between the pulses should be sufficently long to allow the nucleus to return to thermal equilibrium and the disappearence of the NOE effect. The normal delay ($5xT_1$) may not be long enough, hence a delay of $8\text{-}10xT_1$ was chosen.

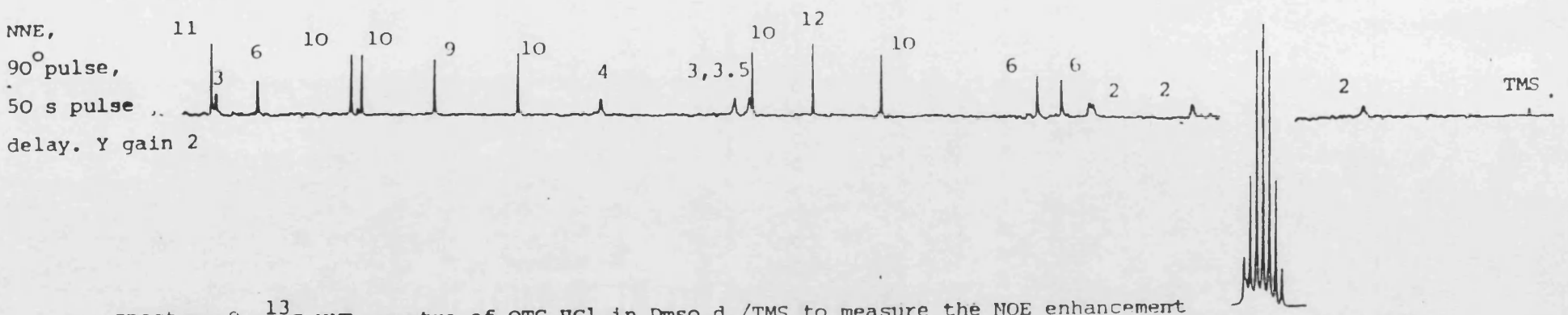
EXPERIMENTAL

OTC HCl was chosen as the best compound due to high solubility in Dmso.d_6 (approximate concentration 100mg/0.5ml).

After much trial and error and using various irradiation modes e.g. complete noise decoupled, no proton irradiation and gated decoupling with 30^o pulse and 1.2s pulse delay, the following two irradiation modes were chosen (number of scans accumulated = 1500).

a) Gated decoupling (NNE), 90^o pulse with 50s pulse delay
b) Complete decoupled spectrum (COM), 90^o pulse with 50s pulse delay

The results are shown in Spectrum 9 and Table 11 Excellent signal to noise (S/N) ratios were achieved, as shown. A value of NOE enhancement (η) was calculated to be 3.18 (neglecting resonances 2, 10, 16,and 17 with NOE factors of 7, 5.6, 6.5 and 6.5 respectively). Signficant broadening observed for resonance lines originating from carbons with α -hydrogens is persumed to be a consequence of rapid relaxation (Wehrli, 1974). The height measurements used in calculating the NOE enhancement factor, are listed in Table 11.



spectrum 9. ^{13}C NMR spectra of OTC HCl in Dmso.d_6/TMS to measure the NOE enhancement factor (η). Peak intensity measurements are in mm

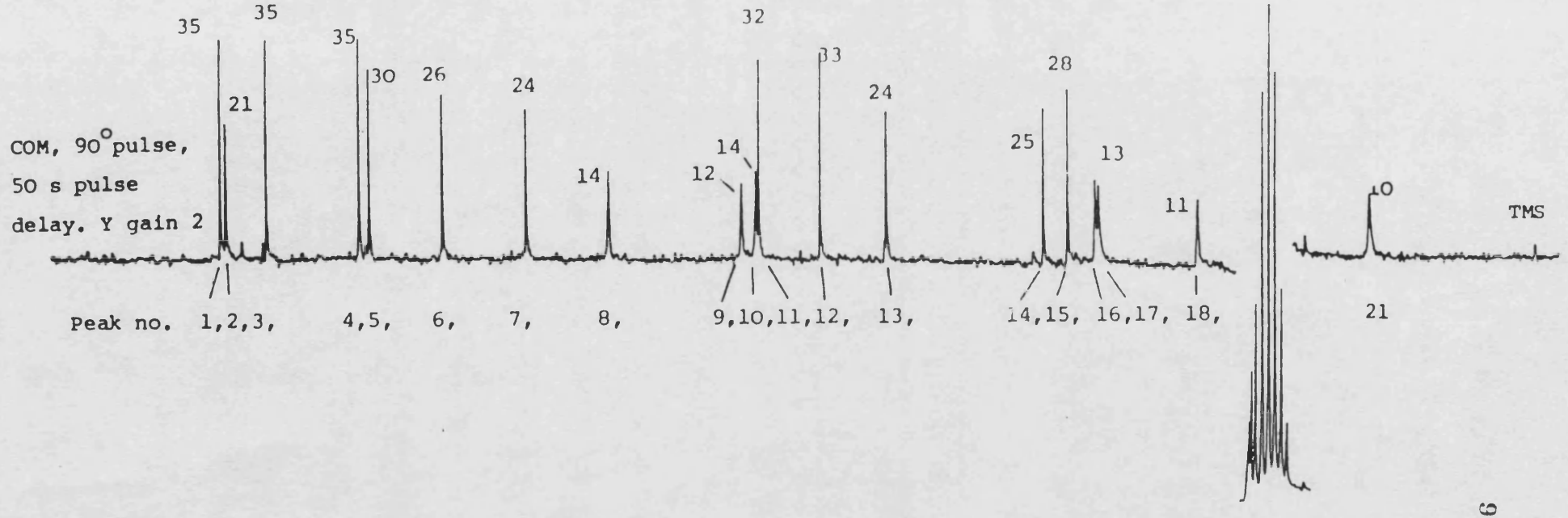


Table 11. Peak height measurements (mm) used to calculate the NOE enhancement factor using (a) NNE with 90° pulse, 50s pulse delay and 1500 scans; (b) COM with 90° pulse, 50s pulse delay and 1500 scans. The solvent used is Dmso.d_6/TMS.

Peak no. (see spectrum 9)	NNE (a)	COM (b)	NOE enhancement (η)
1	11	35	3.18
2	3	21	7.0
3	6	35	5.83
4	10	35	3.5
5	10	30	3.0
6	9	26	2.89
7	10	24	2.4
8	10	14	1.4
9	3	12	4.0
10	3	10	3.33
11	10	32	3.2
12	10	33	3.3
13	10	24	2.4
14	6	25	4.16
15	6	28	4.67
16	2	13	6.5
17	2	13	6.5
18	2	18	9.0
21	2	10	5.0

CONCLUSIONS

The results obtained in the above experiment justify the assumption that relaxation of the tetracyclines ^{13}C nuclei is predominantly via dipole-dipole interactions.

MEASUREMENT OF THE T_1 RELAXATION TIMES

INTRODUCTION

The T_1 measurement programme was carried out on TC HCl and other members of the tetracycline family. The solvents used were 1) Dmso.d_6/TMS or 2)H_2O with D_2O capillary. There are two common methods for determining the spin-lattice relaxation times:

1) Inversion recovery method

2) Progressive saturation technique

In the present study only the first method was employed. The sequence of pulses and pulse delays is as shown in Fig. 10.

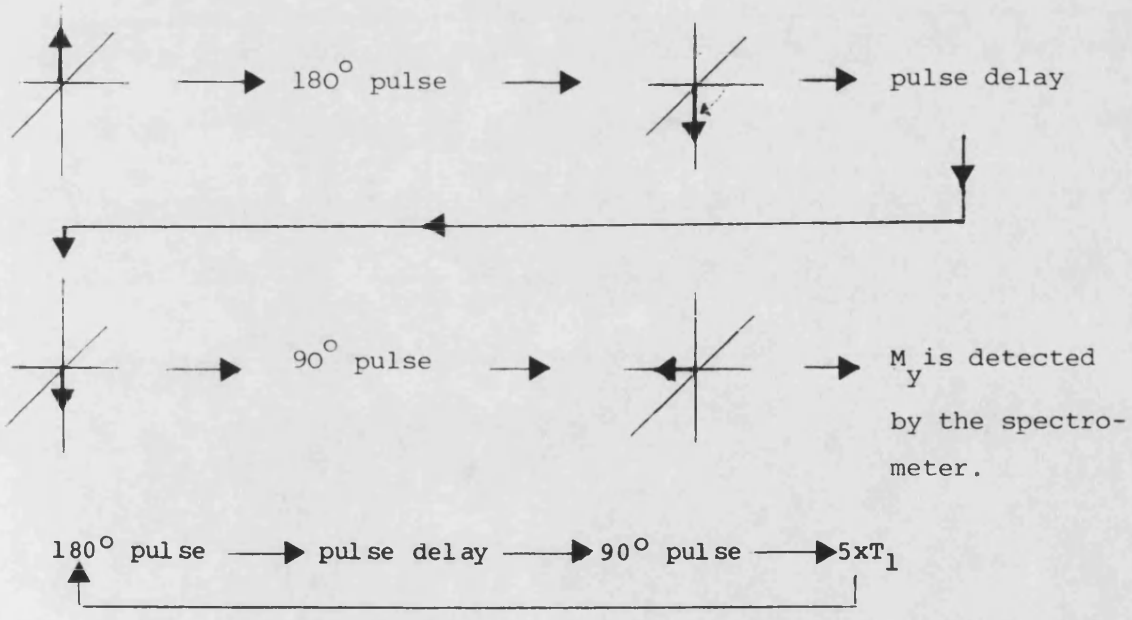


Fig. 10 Sequence of pulses and pulse delays for the inversion recovery method.

Initially a 180° pulse is applied to invert the magnetisation to the z-axis. The magnetisation vector, M_z equals $-M_z$. M_z begins to relax back to its original position M_z via the z-axis. But since the spectrometers can not detect any signal along the z-axis, after a suitable delay, a 90° pulse is applied which flips the remaining magnetisation vector to the y-axis. This is recorded by the spectrometer. T_1 values are calculated by the computer using the mathematical relationship:

$$\ln (M_z - M_z^o) = -\ln (2 M_z^o) - \tau/T_1 \quad \text{.....(4)}$$

A plot of $\ln (M_z - M_z)$ against τ will give a straight line with a gradiant of $-1/T_1$. A rough estimate of the T_1 value is possible from the T_1 spectrum when $M_z=0$.

A single pulse is rarely sufficent to detect the ^{13}C signals at natural abundance and so the cycle must be repeated. In practice, several hundred scans are necessary, depending on the molecular weight and solubility of the molecule.

RESULTS AND DISCUSSION

The T_1 spectral results for the teracyclines are now discussed. Some of these assignments are also discussed in the general assignments of the ^{13}C chemical shifts (see later).

TETRACYCLINE HYDROCHLORIDE (TC HCl)

Pulse delays: 10, 5.5, 3, 1.8, 1.0, .5, .25, .15, .10, .07, .05 secs.

Solvents:

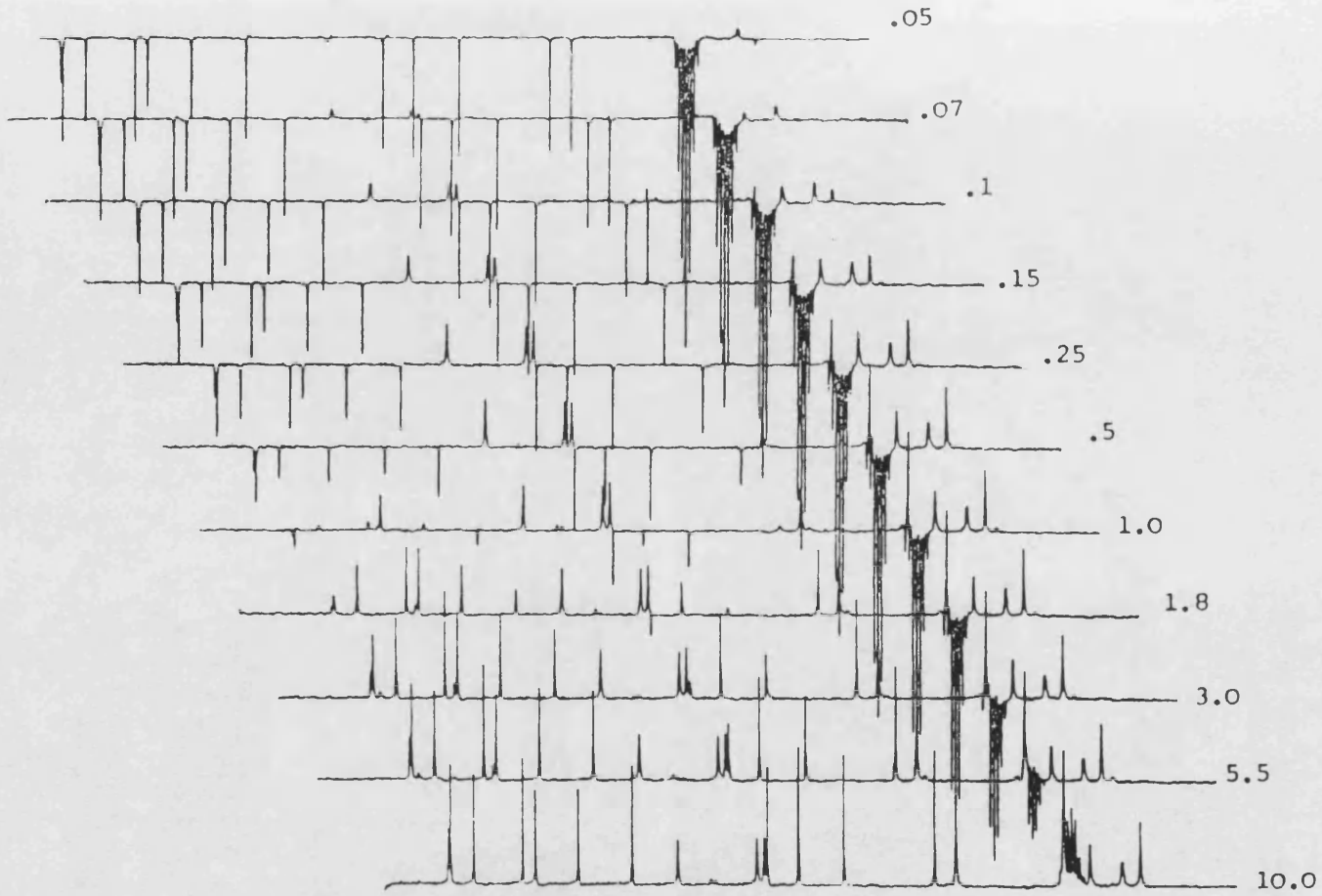
1) Dmso.d_6/TMS

A total of 18 resonances were resolved, two were obscured by the solvent multiplet and two resonances (C_4 and C_6) were overlapping.

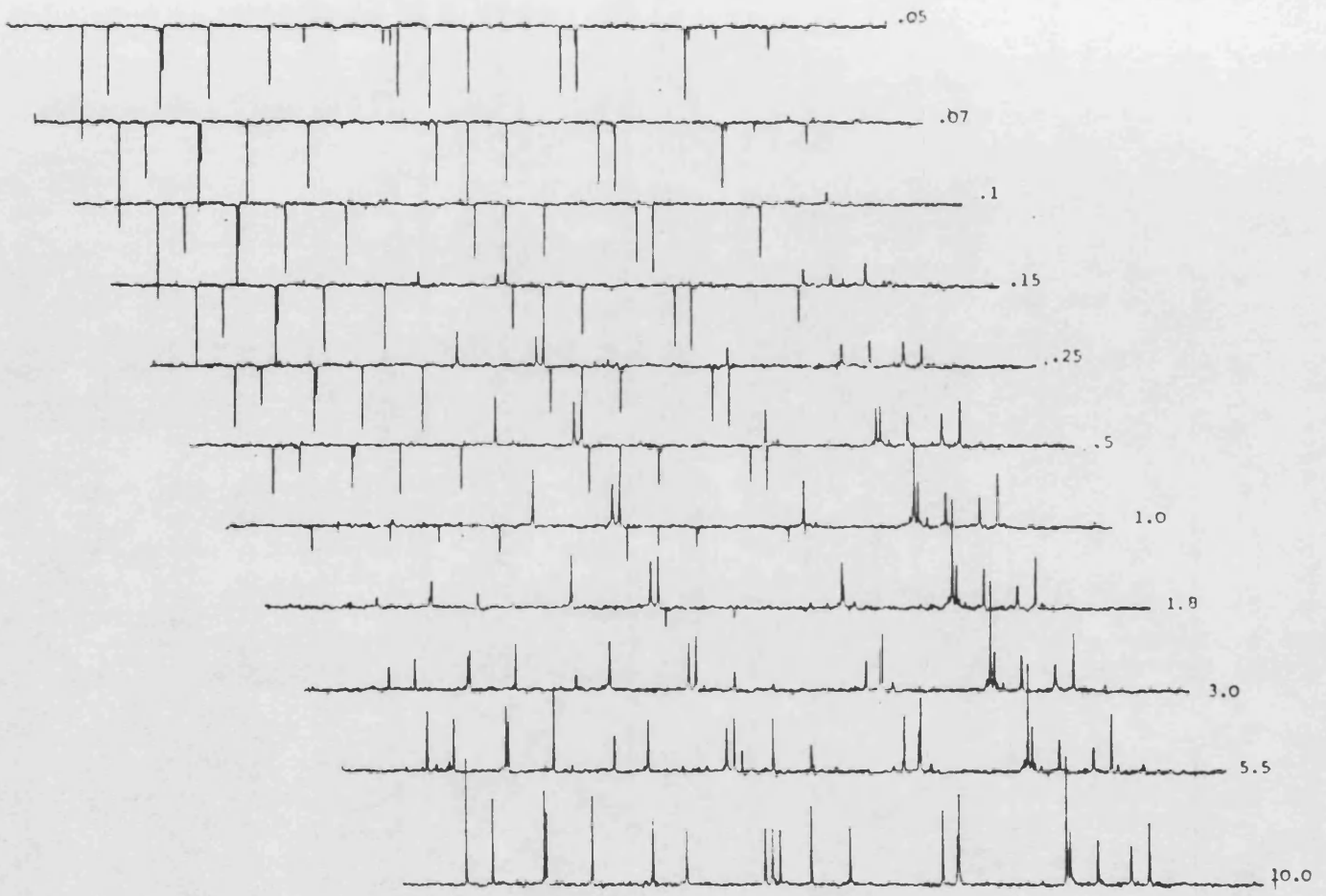
2) H_2O/D_2O capillary

This was a much better solvent mainly due to the absence of any solvent mutiplet. 20 signals are clearly observed with two resonances in the extreme downfield region (193.8 ppm) overlapping. The T_1 values measured in both solvents are listed in Table 12 along with the proton environment of individual carbons and the two spectra are shown in Spectrum 10 and 11.

Although the actual T_1 values differ for corresponding carbons, with results in Dmso.d_6 being significantly lower than those in H_2O, relative rankings are, on average, the same. The difference is assumed to be due to the greater viscosity of Dmso.d_6 (Levy et. al. 1980). As the molecules are spinning in the viscous solvent, they



Spectrum 10. Spin-lattice relaxation spectrum of TC HCl in Dmso.d_6/TMS



Spectrum 11. Spin-lattice relaxation spectrum of TC HCl in H_2O/D_2O capillary

experience variations in the local magnetisation field. As soon as the resonant condition is reached excitation and subsequent relaxation follows. But in a less viscous solvent, the molecules are spinning at a much faster rate and hence take longer to relax.

JUSTIFICATION OF ASSIGNMENTS

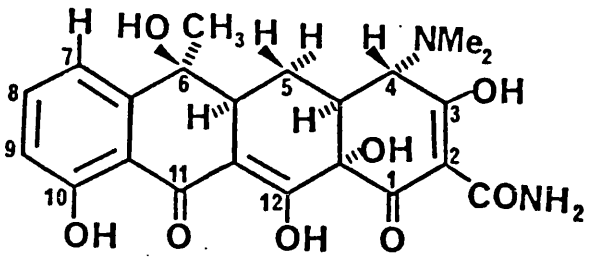
As discussed earlier, the presence of α-hydrogens has a dramatic effect on the relaxation times of a particular carbon i.e. a carbon with α-hydrogen will relax faster than one without the α-hydrogen. In the absence of α hydrogens, β and γ protons also affect the relaxation process.

a) Cq carbons

C_6, C_4 and C_{12a} (38) have resonances at 70.4, 70.7 and 74.4 ppm (in Dmso.d_6). Assignment of the C_{12a} (longest T_1 value of the group) is supported by the fact that it has only two β-hydrogens whereas C_6 has five. The difference is less pronounced in H_2O, but the lowest field resonance still has the longest T_1 value. The 70.7 ppm resonance is clearly due to C_4 (T_1= 0.21 s) due to the presence of one α-hydrogen.

TC HCl	T_1	T_1	Assignment	Hydrogen environment			
ppm	Dmso.d_6	H_2O		α	β	γ	δ
193.4	2.27	3.03	11	-	-	1	6
192.9	2.20	3.03	1	-	-	2	7
187.2	1.48	2.63	3	-	2	1	10
175.0	1.30	2.35	12	-	1	3	3
172.1	0.87	1.85	$CONH_2$	-	2	1	1
161.3	1.27	2.17	10	-	2	1	1
147.9	2.17	3.20	6a	-	1	6	4
136.5	0.09	0.14	8	1	2	-	1
116.9	0.09	0.17	7	1	1	1	5
115.2	0.09	0.15	9	1	1	2	-
114.4	3.9	4.58	10a	-	-	3	6
106.8	2.09	2.97	11a	-	1	3	6
95.7	2.83	3.77	2	-	-	4	2
73.1	1.54	2.39	12a	-	2	4	1
67.9	----	0.21	4	1	1	9	2
67.9	1.06	2.22	6	-	5	3	2
42.0	0.87	2.53	5a	1	2	5	3
42.0	----	0.60	NMe_2	6	-	1	2
35.3	----	0.13	4a	1	3	2	8
26.9	----	0.16	5	2	2	1	5
22.5	0.12	0.20	C_6-methyl	3	-	2	3

Table. 12 T_1 relaxation times of TC HCl in Dmso.d_6 and H_2O along with their assignments and the proton environment.



(38)

LOW-FIELD RESONANCES

The two lowest field resonances (both near 193 ppm in Dmso.d_6) and assigned to C_{11} and C_1, have very similar T_1 values. But the C_3 resonance (187.2 ppm) possesses a much lower T_1 value due to the two β-hydrogens (Table 12). The C_{12} and $CONH_2$ resonances also correlate the observed T_1 values with the proton environment as do those of C_{10}, C_{6a}, C_{10a}, C_{11a}, C_2 and C_{12a}.

PROTONATED CARBONS

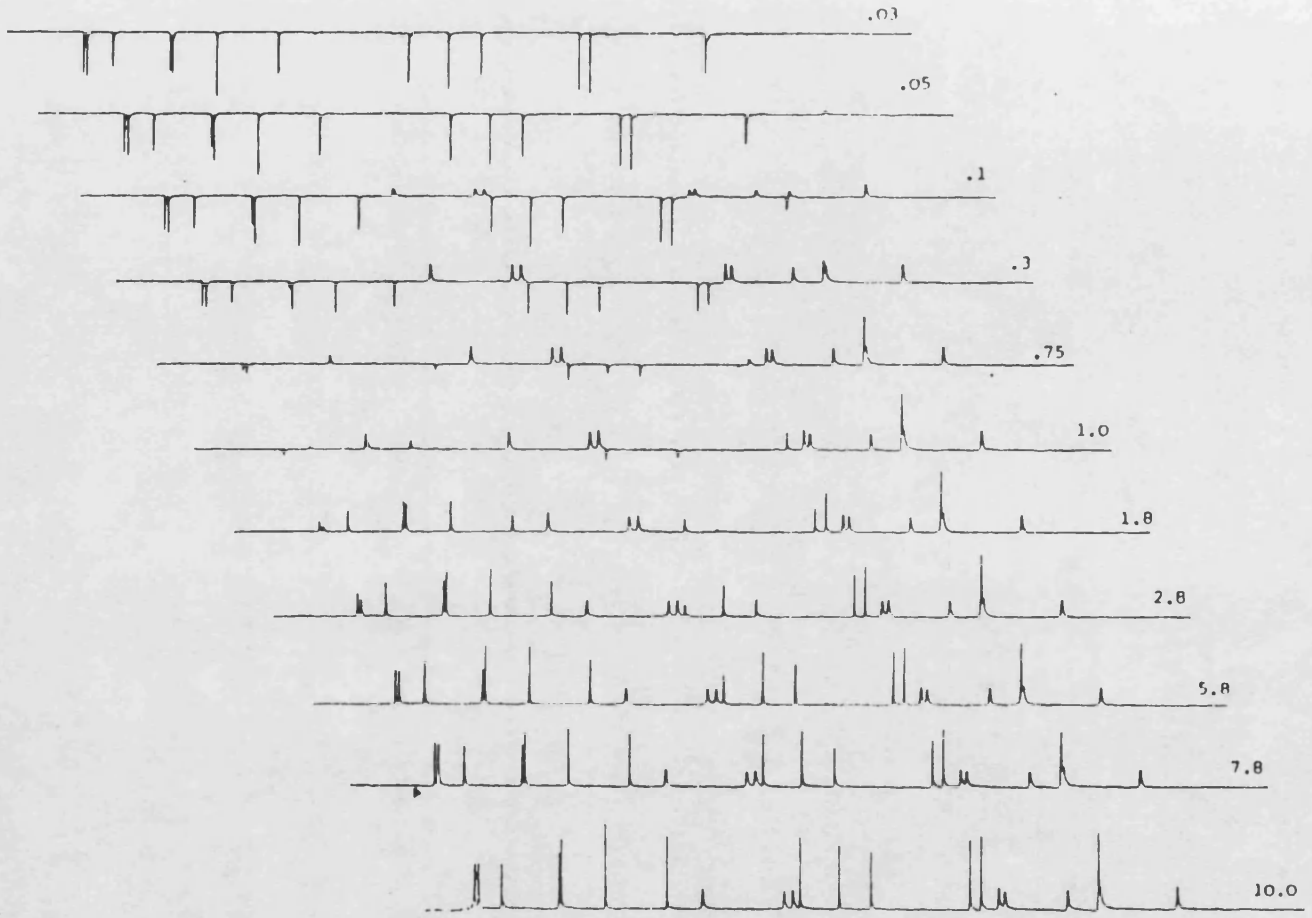
C_7, C_8 and C_9 resonances are observed at 118.7, 138.5 and 116.8 ppm respectively. These three carbons have very short T_1 values (of the order 0.09 s in Dmso.d_6 and 1.5 s in H_2O) due to the presence of α-hydrogens.

All high field signals (< 70 ppm), due to the protonated carbons, have T_1 values less than 0.3 s, except for one signal within the solvent band which is most probably due to the NMe_2. The NMe_2 carbons may relax by a spin-rotation mechanism since, in the preferred stereochemical configuration, the NMe_2 is not restrained within the tetracycline skeleton. As this process is less efficent than dipole-dipole relaxation (Abraham and Loftus, 1980), large T_1 values are to be expected. Spin-rotation contribution to the relaxation of C_6-CH_3 is to a much lesser extent as judged by its low T_1 value. This may be due to the fact that CH_3 is in a more hindered state than the NMe_2 carbons and is hence less free to spin about the C_6 bond.

OXYTETRACYCLINE HYDROCHLORIDE (OTC HCl)

Pulse delay: 10, 7.8, 5.8, 2.8, 1.8, 1.0, .75, .3, .1, .05, .03 secs.
Solvents:
H_2O/D_2O capillary (Spectrum 12)
The T_1 values are listed in Table 13 with proton environment of each carbon.

The C_6-methyl of OTC HCl is even more hindered than that of TC HCl, hence the spin-rotation contribution is expected to be even lower. This correlates with a very short T_1 value (0.06 s) indicating that its main mechanism of relaxation is via the dipole-dipole interaction.



Spectrum 12 Spin-lattice relaxation spectrum of OTC HCl in H_2O/D_2O capillary

OTC HCl	T_1	Assignment	Proton environment			
ppm	D_2O		α	β	γ	δ
193.7	2.40	11	-	-	1	5
192.9	1.97	1	-	-	2	6
187.1	1.32	3	-	2	1	9
173.5	1.55	12	-	1	3	2
171.9	1.01	$CONH_2$	-	2	1	1
161.2	1.29	10	-	2	1	1
148.7	2.32	6a	-	1	6	3
136.6	0.09	8	1	2	-	1
118.9	0.08	7	1	1	1	5
114.9	0.09	9	1	1	2	-
114.4	3.07	10a	-	-	3	6
105.3	1.82	11a	-	1	2	7
95.4	2.54	2	-	-	4	2
72.5	1.44	12a	-	2	3	2
64.9	0.09	4	1	1	8	3
68.9	1.02	6	1	1	8	3
63.2	0.07	5	1	3	1	5
49.8	0.09	5a	1	1	6	3
41.8	0.33	NMe_2	6	-	1	1
42·0	0.10	4a	1	2	3	8
24.6	0.06	C_6-methyl	3	-	2	2

Table 13. T_1 relaxation times of OTC HCl in D_2O along with their assignments and the proton environments.

All the other rankings are in accordance with the assignments of OTC HCl.

DOXYCYCLINE HYCLATE

Pulse delays: 10, 7.8, 5.8, 2.8, 1.0, .75, .3, .1, .05, .03 secs

Solvent: Dmso.d_6/TMS

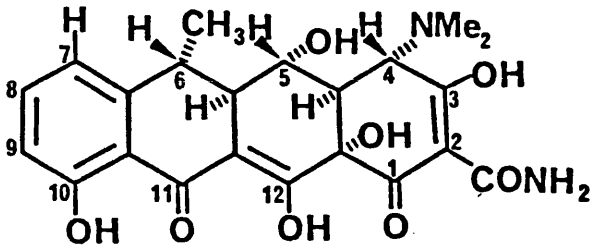
T_1 values are listed in Table 14.

Since two peaks in the upfield region belong to ethanol, they should be assigned beyond any doubt. Due to the small size of the ethanol molecule, it would be spinning at a much faster rate than the much bulkier tetracycline skeleton, hence much longer T_1 values are expected for the two ethanol peaks. The two peaks at 56.0 and 18.4 ppm show very large T_1 values (of the order 3.5 s).

C_{10} and C_{6a} now have comp-rable T_1 values (0.93 and 1.0 s) unlike the same carbons in TC HCl. In doxycycline, C_{6a} now has one extra β -hydrogen, hence both C_{6a} and C_{10} have similar proton environment (39).

doxycycline	T_1 (s)	Assignment
193.6	1.60	11
192.6	1.53	1
187.4	1.06	3
173.5	1.02	12
171.8	0.58	$CONH_2$
161.2	0.93	10
147.8	1.00	6a
136.7	0.05	8
115.9	0.02	7
115.6	0.02	9
115.5	2.22	10a
107.2	1.29	11a
95.3	1.37	2
73.1	1.07	12a
68.1	0.05	5
64.7	0.04	4
45.2	0.07	5a
42.0	0.05	6
42.0	0.06	NMe_2
42.0	0.07	4a
15.0	0.07	C_6-Me

Table 14 T_1 relaxation times of doxycycline in Dmso.d_6/TMS with their assignments. Underline resonances were obscured by the solvent multiplet.



(39)

The rest of the data correspond with the proton environments of individual carbons.

Section 2 ^{13}C NMR

Theoretical aspects

Sensitivity problems

^{13}C-NMR SPECTROSCOPY

THEORETICAL ASPECTS

The normal range of ^{13}C chemical shifts is 0-200 ppm, so that the spread of chemical shifts is about twenty times that of protons. Reasons for this are complex, but are considered to be chiefly due to the fact that paramagnetic shielding effects are dominant in ^{13}C nuclear shielding rather than the diamagnetic as in ^{1}H NMR (Stothers, 1972). It is appropriate, at this point, to include a brief discussion of the theoretical aspects of ^{13}C-NMR.

Consider the s electrons in a molecule. These electrons are spherically symmetrical and circulate in the applied magnetic field. Since a circulating electron is an electric current, it produces a magnetic field at the nucleus which opposes the external field. Thus

in order to obtain the resonant conditions ($\nu = \frac{\gamma B}{2\pi}$) (where γ = magnetogyric ratio, B = applied magnetic field, ν = frequency

of radiation), it is necessary to increase the external field over that for the isolated nucleus. If B_{ext} is the applied field and B_o is the field at the nucleus, the the nuclear shielding (ΔB) is given by:-

$$\Delta B = B_{ext} - B_o \qquad \text{.....(5)}$$

This upfield shift of the nucleus is called a diamagnetic shift, and since every molecule has s electrons this phenomenon (known as diamagnetism) makes a universal contribution.

For electrons in p-orbitals and all other non-spherical orbitals, there is no spherical symmetry. Electrons in these orbitals produce large magnetic fields at the nucleus, which when averaged over the molecular motions give a low field shift which augments the applied field. This deshielding is called the paramagnetic shift. The proton (^1H) is a special case, as it is the only molecule with no p-electrons and therefore there is no paramagnetic term from its own valency electrons. This is the fundamental reason for the small range of proton chemical shifts (0-10 ppm) when compared with all other nuclei which have p-electrons and shift ranges of 0-200 ppm or more.

Furthermore, the effect of substituents on ^{13}C shifts is not confined to the nearest atom, as is essentially the case for proton shifts, but the effects of substituents two, three and four bonds from a particular carbon must also be considered. In general, the screening constant (σ), as in

$B_i = B_o (1- \sigma)$ .....(6)

B_i= magnetic field required for resonance

B_o= applied field

is considered to be a summation of three components.

$\sigma = \sigma_d + \sigma_p + \sigma_i$ .....(7)

σ_d = diamagnetic term; σ_p = paramagnetic term;

σ_i = neighbouring atom term.

Because ^{13}C spectra display resonances over a wide ppm range, almost all carbons of a molecule that differ in environment are likely to give rise to unique and mutually resolvable signals. This is generally the case when the spectra are recorded at 22.5 and 25 MHz. Even greater resolution is achieved when super cooled magnets are employed which allow operations at 67.8and 100 MHz.

SENSITIVITY PROBLEM

One of the main limitations of NMR spectroscopy is its inherent lack of sensitivity relative to other important spectroscopic techniques such as electronic (UV/VIS) and vibrational (IR) spectroscopy. This is due to the small magnitude of the energy changes involved in NMR transitions.

The sensitivity problem becomes important when we consider the

spectra of nuclei other than proton eg. ^{13}C. The main reasoning for this is two fold

1) The natural abundance of the ^{13}C isotope is only 1.1%
2) The magnetogyric ratio (γ) of ^{13}C nucleus is only about one quarter that of ^{1}H nucleus.

Since the sensitivity of a nucleus in a magnetic resonance experiment at constant field is proportional to (γ^3), a ^{13}C nucleus gives rise to 1/64th the signal that a proton would yield. Hence, taking natural abundance into account, a ^{13}C NMR experiment is about 6000 times less sensitive than one involving protons.

One of the simplest ways of overcoming such problems is to record several spectra from a sample and then simply add then together. The NMR signals will add coherently, whereas the noise, being random. will only add as the square root of the number of spectra accumulated. This leads to an overall improvement in signal to noise ratio (S/N).

Prior to 1960s, ^{13}C NMR could only be obtained by conventional frequency or field sweep NMR (also known as continuous wave) in which only one frequency is being observed at any given instant. Experimental advances over the last 10-20 years have led to the pulsed NMR techniques, in which all the ^{13}C nuclei in a sample are excited simultaneously by the application of a short radio frequency (rf)

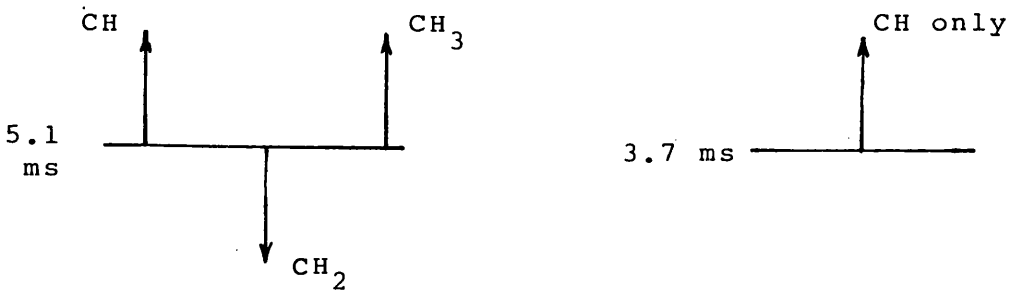
pulse of high power. The respose of a sample to this excitation is absorption of individual frequency components by each nucleus. These frequencies are detected by the receiver to yield a pattern called the free induction decay (FID). The FID corresponding to absorption of one frequency is an exponentially decaying sine wave.

Since the FID is a measurement of intensity as a function of time, it is often referred to as the time domain signal, whereas, the corresponing CW measurement provide a frequency domain signal. It is possible to inter-convert data between the time and frequency domains by means of a mathematical process known as Fourier transformation (FT). Since the time taken for a single experiment is very short (approximately 2s allowing for the pulse itself, acquisition time and pulse delay) many scans may be made over relatively short period of time and the data time averaged to provide the satisfactory S/N ratios in the final spectrum.

The normal ^{13}C-NMR spectrum is generally recorded in the proton decoupled mode (COM). This has the effect of simultaneously decoupling all the protons in the molecule. Hence, all carbons except the equivalent ones, show up as single resonances. Apart from the increase in S/N ratio that results from the collapse of the multiplet structure, proton noise decoupling gives rise to an additional increase in intensity due to the Nuclear Overhauser Effect (NOE), which in some cases produces an almost three fold increase in intensity. Although proton noise decoupling produces a considerable

simplification in the appearance of ^{13}C spectra, it also removes all the coupling information so that despite their straightforward appearance, fully decoupled spectra may be difficult to assign. This is overcome by use of the off-resonance (OFR) mode, in which only the $^{1}J_{CH}$ couplings (H attached directly to C) are allowed (and these in reduced magnitude). Hence, primary carbons (bearing three hydrogens) will appear as quartets, secondary carbons as triplets, tertiary carbons as doublets and quaternary carbons as singlets thus permitting immediate classification into each of these four types.

Most of the NOE obtained in the proton noise decoupled spectrum is also retained in the OFR spectrum. A different experimental technique, Insensitive Nuclei Enhanced by Polarization Transfer (INEPT) was introduced in 1983 (Morris and Freeman, 1983). With this programme the resonances are not split into multiplets, instead in one mode the CH and CH_3 are positive and CH_2 are negative peaks, whereas in another mode only CH peaks are observed (as shown below). The quaternary carbons do not show up at all.



Further information about COM, OFR and NOE may be obtained from

published literature (Abraham and Loftus, 1980). In certain cases the spectral assignments of this thesis have been corroborated by consideration of spin-lattice relaxation times (T_1) (see page 88).

Section 3 ^{13}C NMR ANALYSIS OF SPECTRA

Aim and approach

Analysis of ^{13}C NMR spectrum of TC HCl

- Quartets
- Triplets
- Doublets (low field)
- Doublets (High field)
- Singlets, C_6 and C_{12a} singlets
- Lowest-field resonances
- Amide carbonyl
- Quaternary carbons linked to oxygen
- Quaternary carbons without oxygen

Tetracycline base

Chlortetracycline hydrochloride

6-demethyl chlortetracycline HCl

Minocycline

Oxytetracycline HCl

Methacycline

Meclocycline

6-deoxy oxytetracycline and its 6-epi isomer

Semi-synthetic tetracyclines, lymecycline, rolitetracycline

ANALYSIS OF ^{13}C NMR SPECTRA OF TETRACYCLINE HYDROCHLORIDE AND ITS ANALOGUES

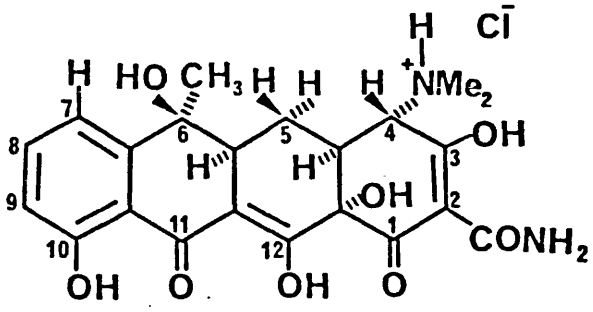
AIM AND APPROACH

A detailed study of the assignments of the ^{13}C NMR spectrum of TC HCl will be given, this derivative being regarded as the parent member of the group. Spectra of analogues will then be examined and assigned on the basis of similarities to and differences from the parent structure.

Although some NMR studies based on ^{13}C have been reported (Wittenau, 1964; Mazzola et al. 1980), the most comprehensive report was that of Asleson and Frank (1975) covering tetracycline and some of its derivatives. The aim of the present work is to analyse the spectral features of the tetracycline group of antibiotics incorporating the original arguments of Asleson and Frank (1975). Degradation products and semi-synthetic tetracyclines e.g lymecycline and rolitetracycline will also be studied. Some of the spectral assignments are supported by relaxation time (T_1) measurements which are more fully discussed on page 102.

To assist in assignment of the tetracyclines, use is made of models or standard compounds. The assignment of these models is discussed in chapter 5.

ANALYSIS OF ^{13}C NMR SPECTRUM OF TC HCl



(3) TC HCl

There is a total of 22 carbon atoms in the TC HCl molecule (3), but the two methyl carbons attached to the nitrogen are chemically and magnetically equivalent, and therefore produce only one signal.

Hence a total of 21 signals are expected provided

1) all different carbons give distinct resonances

2) there is minimum or no overlapping between the signals.

The chemical shifts for TC HCl are presented in Table 15 , and the completely decoupled spectra run in Dmso.d_6/TMS and D_2O is shown in Spectrum 13.

Resonances due to 19 of the 21 different types of carbon are apparent in the ^{13}C NMR spectrum run in Dmso.d_6. In the same spectrum two resonances overlap at 67.9 ppm which are resolved in the spectrum run in D_2O.

Spectrum 13. Proton noise decoupled spectrum (^{13}C NMR) of TC HCl in (a) Dmso.d_6 and (b) H_2O. The assignments given apply to both spectra. The broad nature and relatively low intensities of resonances C_8, C_7, C_9, C_{4a} and C_5 (due to protonated carbons) in spectrum (a) should be noted, as should the fact that disparities in signal intensities of resonances C_{6a} through C_2 are much smaller in spectrum (b). The NMe_2 and C_{5a} resonances, obscured by the solvent multiplet in spectrum (a), are clearly resolved in spectrum (b).

Table 15 ^{13}C chemical shifts and spin lattice relaxation times (T_1) of TC HCl in (a) Dmso.d_6, (b) D_2O. (TMS = 0 ppm) Underlined resonances are obscured by solvent multiplets

	TC HCl (ppm)		T_1 (s)	
Position	(a)	(b)	(a)	(b)
1	192.9	193.8	2.20	3.03
2	95.7	97.5	2.83	3.77
3	187.2	187.2	1.48	2.63
4	67.9	70.4	1.54	0.21
4a	35.3	35.4	----	0.13
5	26.9	26.9	----	0.13
5a	42.0	42.3	0.87	2.53
6	67.9	70.7	1.06	2.22
6a	147.9	146.9	2.17	3.20
7	116.9	118.7	0.09	0.17
8	136.5	138.5	0.09	0.14
9	115.2	116.8	0.09	0.15
10	161.3	162.0	1.27	2.17
10a	114.4	114.9	3.90	4.58
11	193.4	193.8	2.27	3.03
11a	106.8	107.1	2.09	2.97
12	175.0	174.1	1.30	2.35
12a	73.1	74.4	2.83	2.39
$CONH_2$	172.1	173.5	0.87	1.85
C_4-NMe	42.0	43.3	----	0.16
C_6-Me	22.5	22.3	0.12	0.20

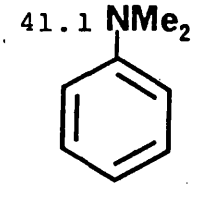
The 21 resonances of TC HCl may be subdivided into the following groups:-

Singlets	C-1,2,3,6,6a,10,10a,11,11a,12,12a,$CONH_2$	= 12
Doublets	C-4,4a,5a	= 3
	C-7,8,9	= 3
Triplets	C-5	= 1
Quartets	NMe_2, C_6-Me	= 2
	Total	= 21

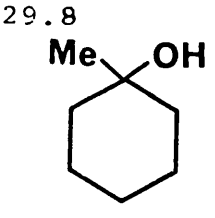
The assignment of these groups is now discussed.

QUARTETS

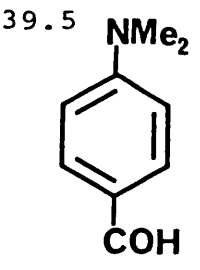
Two quartets are expected due to C_6-methyl and C_4-NMe_2. One off-resonance quartet was observed at 22.5 ppm, while the second multiplet was obscured by the solvent resonances. The assignment of the 22.5 ppm resonance to C_6-methyl and the lower field signal (42 ppm) to C_4-NMe_2 is justified on the grounds of greater electronegativity of nitrogen compared with carbon and also confirmed by the chemical shift data of the reference compounds shown below.



(42)



(43)

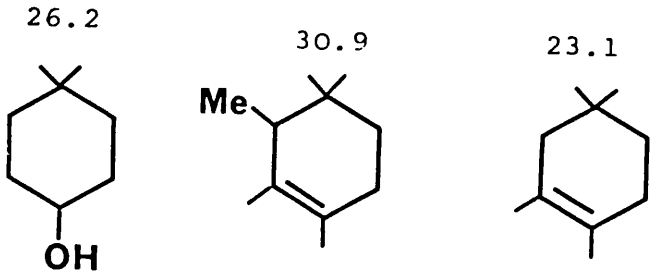


(44)

(Roberts et al. 1970)

TRIPLETS

Only one is expected due to C_5. Thus the triplet observed in the off-resonance spectrum at 26.9 ppm must be due to the C_5 carbon. This assignment is confirmed by reference to the chemical shift data of the model compounds (45).



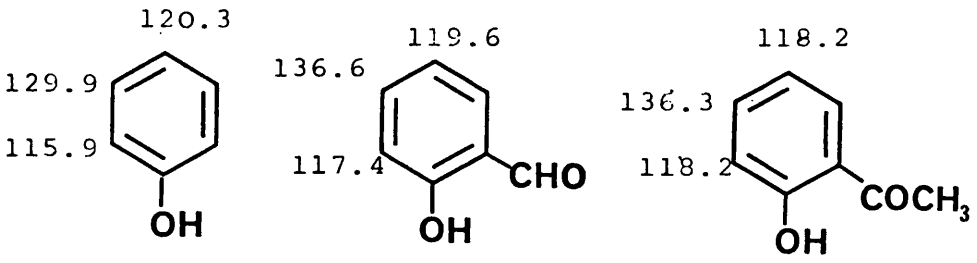
(45) (Stothers, 1972)

DOUBLETS (LOW FIELD)

All resonances due to protonated carbons are broad and of low intensity compared with most quaternary carbons signals, a fact attributed to the very fast relaxation rates of protonated carbons (see Table 15), which operate in the viscous solutions necessary for spectral measurements. These resonances are narrower and more intense in spectra of aqueous solutions of reduced viscosity. This observation is common to all tetracycline spectra, and may be put to advantage in that it allows the ready identification of the C_7, C_8 and

C_9 resonances of ring **D** (typified in Spectrum 13).

Assignments for C_7, C_8 and C_9 are confirmed on the basis of comparison with the model compounds (46).

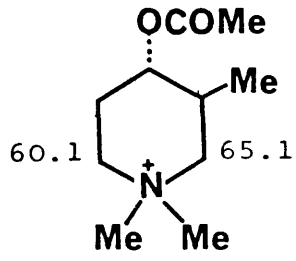


(46) (^{13}C data bank, 1976)

Within this phenolic trio, C_8 is assigned to 136.5 ppm, but C_7 and C_9 shifts are very close together and may be interchanged.

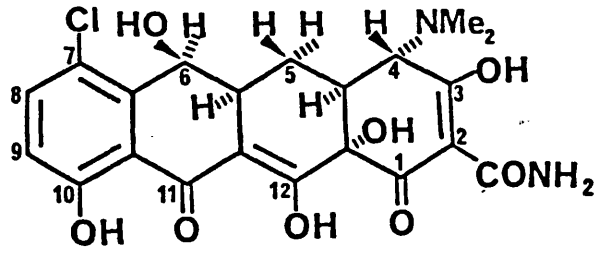
DOUBLETS (UPFIELD)

Of the three resonances assigned to C_4, C_{4a} and C_{5a} (OFR doublets), that at lowest field (67.9 ppm) is assigned to C_4 since this carbon is subject to α-deshielding by nitrogen and to several α/β effects due to surrounding carbon atoms (cf. α-carbons shifts of model (47)



(47) Casy, 1971

Resonances due to C_{4a} and C_{5a} may be assigned by comparison with he corresponding chemical shifts of 6-demethyl CTC (4).



(4) 6-demethyl CTC HCl

Due to the loss of β -deshielding influence of C_6-methyl, the C_{5a} resonance of 6-demethyl CTC should be higher field than the corresponding chemical shift in TC, whereas, the C_{4a} chemical shift should be

similar in both the compounds. The 6-demethyl CTC spectrum showed a doublet near 35 ppm (similar value to the C-H doublet of TC) and one near 37 ppm (higher field than the 43 ppm doublet of TC). Hence, the assignments of the upfield doublets are confirmed as follows:-

	Dmso.d_6	H_2O/D_2O capillary	
C_4	67.9	70.4	
C_{4a}	35.3	35.4	
C_{5a}	<u>42.0</u>	<u>42.3</u>	underlined resonance obscured by Dmso.d_6 multiplet.

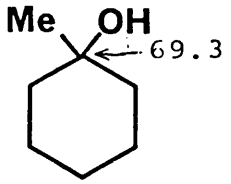
SINGLETS

A total of 12 singlets are expected due to the quaternary (Cq) carbons:-

C-1,2,3,6,6a,10,10a,11,11a,12,12a and $CONH_2$

C_6 AND C_{12a} SINGLETS

The high field singlets of C_6 and C_{12a} are considered first. Singlet resonance at 67.9 ppm is assigned to C_6 (overlapps C_4 signal) since it falls within the C_q range 70-90 ppm of tertiary alcohols (cf. model compound 43)



(43) (Stothers, 1972)

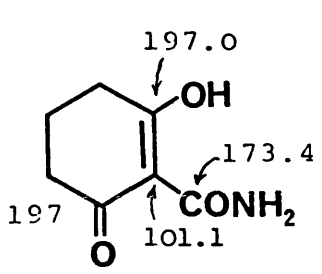
The resonance at 73.1 ppm is assigned to C_{12a}. Supportive evidence for the lower field resonance may be obtained from the corresponding chemical shift data of 6-deoxy OTC, 6-demethyl CTC and methacycline.

doxycycline	C_{12a} = 73.0 ppm
6-demethyl CTC	" = 73.7 ppm
methacycline	" = 73.5 ppm

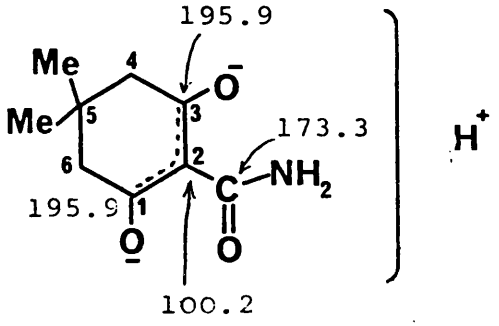
Since the C_{12a} carbon is well removed from these C_6 structural changes, the chemical shift should be similar in all these tetracycline analogues. Therefore C_{12a} is assigned to 73.1 ppm.

LOWEST FIELD RESONANCES

The lowest field pair (193.4 and 192.9 ppm) is assigned to carbonyl carbons at C_{11} and C_1, by reference to the model data on α , β - unsaturated ketones and as judged from data on the models (48) and (49).



(48)



(49)

(Asleson and Frank, 1975)

A decision on the exact assignemnt of C_1 and C_{11} was reached by monitoring the signal as a funtion of pH (Asleson and Frank, 1975). The 192.9 ppm resonance shifted at pH 3.0 as did that 187.2 ppm. Therefore the 192.9 ppm resonance must be due to the carbon of ring A (i.e. C_1) since ring A is known to be the first pK_a site (Fig.11) The 193.3 ppm resonance did not shift until the pH exceeded 7.0, and was therefore assigned to C_{11}. The same pH study allows assignment of the next higher field resonance (187.2 ppm) to C_3 as confirmed by comparative T_1 values (see page 102) : C_{11} 2.27s, C_1 2.2s, C_3 1.48s in Dmso.d_6

AMIDE CARBONYL

By reference to the model compounds (48 , 49), either the 172.1 or 175.0 ppm (Dmso.d_6) resonance may be assigned to the amide carbon of TC HCl.In support only one resonance is observed in the 170-180 ppm

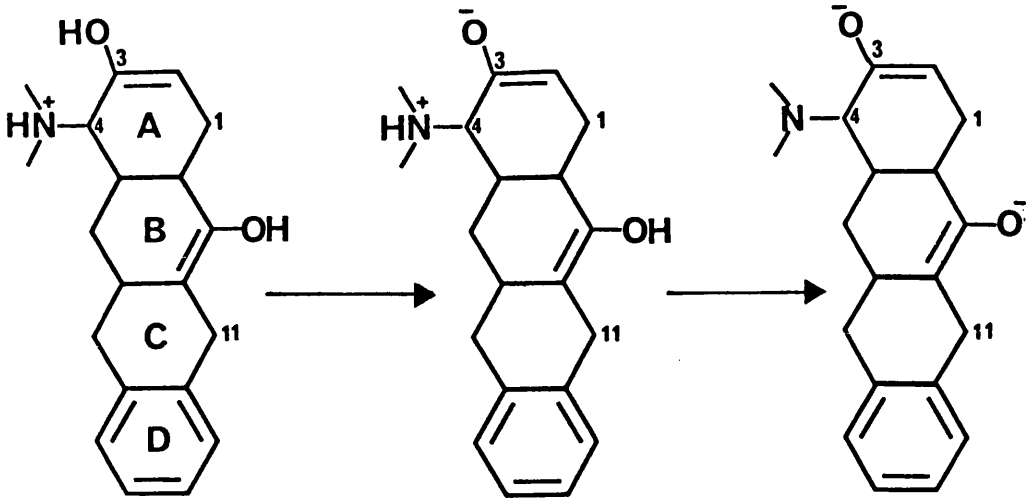
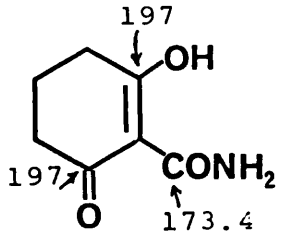
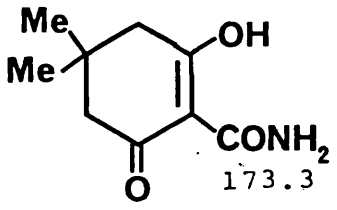


Fig.11 Ionisation stages of tetracycline as a function of pH (Knox and Jurand, 1979). Only ionisable groups are shown.

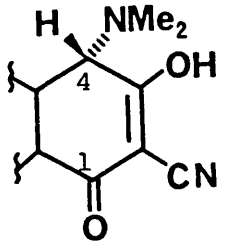


(48) Asleson, 1975



(49)

spectral range of the 2-cyano analogue of TC HCL (22).



TC HCl	172.1, 175.0 ppm
2-cyano TC	119.2, 178.7

(22) partial structure of
2-cyano TC

Choice of the higher field resonance (at 172.1 ppm) as due to the amide carbonyl, rests upon the corresponding model compound data, but more convincingly on the comparative T_1 values for the 172.1 and 175.0 ppm resonances (see page 102).

There are six carbons still left to be assigned. These may be conveniently subdivided into two groups:-

1) quaternary carbons with oxygen: C_{10}, C_{12}

2) " " without Oxygen: C_2, C_{6a}, C_{10a}, C_{11a}

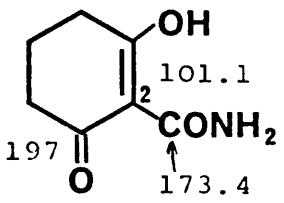
QUATERNARY CARBONS LINKED TO OXYGEN

The two lowest field resonances, 161.3 and 175.0 ppm, must be due to C_{10} and C_{12} due to the strong deshielding influence of oxygen. the 161 ppm value is typical of phenols with ortho placed carbon substituents (*). Therefore, 161.3 ppm may undoubtedly be assigned to C_{10}. Hence C_{12} must give rise to the resonance at 175.0 ppm.

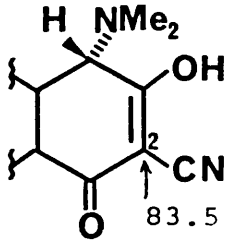
QUATERNARY CARBONS WITHOUT OXYGEN

These are attributable to unsaturated carbons not attached to oxygen i.e. C_2, C_{6a}, C_{10a} and C_{11a}.

The highest field signal of this group (95.7 ppm) is due to C_2 according to the chemical shifts of the model (23).



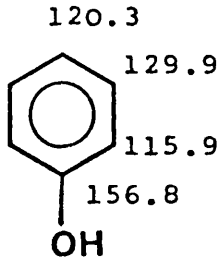
(23) (Asleson, 1975)



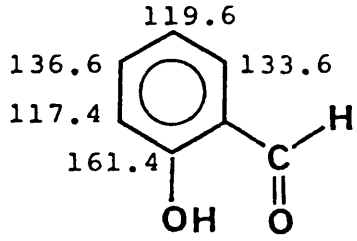
(22) 2-cyano TC

In support, the 95.5 ppm resonance of TC HCl shifts to 83.5 ppm on conversion to the 2-cyano TC derivative (22).

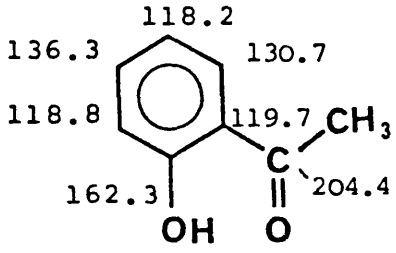
* see page 137



(50)

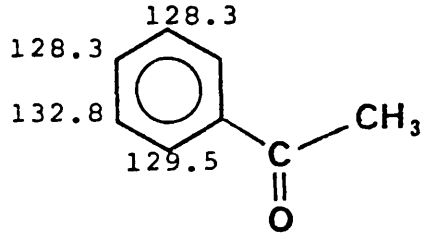


(51) Asleson, 1975

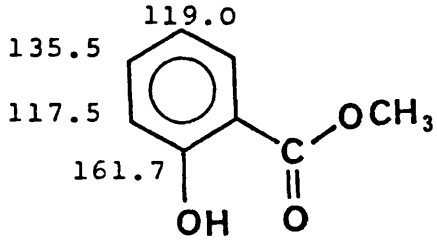


Asleson, 1975

(52)



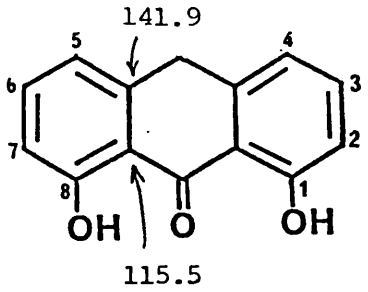
(53)



(54)

(Reference for compounds (53 and 54): ^{13}C data bank, 1976)

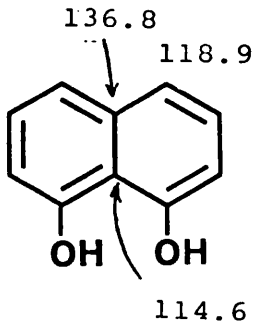
The lowest field resonance of this group (147.9 ppm) may be assigned to C_{6a} on the basis of the model (55), together with the sensitivity of the C_{6a} resonance to structural changes at C_6 and C_7.



(55) dithranol

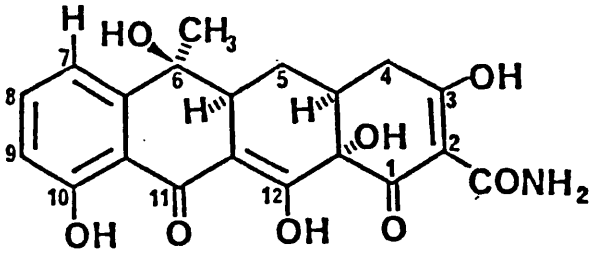
	C_{6a} (Dmso.d_6)
TC HCl	147.9 ppm
CTC HCl	143.6 ppm
6-demethyl CTC HCl	140.6 ppm
Methacycline HCl	142.7 ppm

Of the two remaining Cq resonances, that near 114 ppm is assigned to C_{10a} on the basis of models (55 and 56).



(56)

By elimination, the 106.8 ppm resonance is attributable to C_{11a}. Relaxation time measurements of this quartet support these assignments, in that lower T_1 values were found for the C_{11a} and C_{6a} carbons (each with one β -hydrogen (57) than for the C_2 and C_{10a} carbons which lack β -hydrogens (see page 106). The T_1 values and their contribution to assigning various resonances are discussed in more detail in chapter 3 section 1.

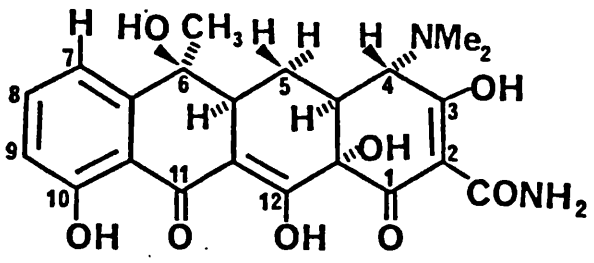


(57) (partial formula)

Having examined the full assignment of the TC HCl in detail, a study of the ^{13}C NMR spectra of its derivatives will now be presented. The approach will be to compare the spectrum of the analogue with that of TC HCl, and to assign resonances of the analogue spectrum directly from the standard spectrum on the basis of structural similarities or differences between the two compounds. Corresponding carbons of TC HCl-variant pair, in areas of the molecule remote from those where structural differences occur, should differ little in environment, hence assignments of such chemical shifts of the variant molecule may be made directly from the TC HCl shift data. It will be seen that many assignments may be made in this way. Chemical shift differences are interpretted in terms of shifts that are to be anticipated from the particular structural differences involved.

TETRACYCLINE BASE

In the spectrum of TC base (58), run in Dmso.d_6/TMS, sixteen resonances downfield and two upfield of the Dmso.d_6 multiplet are observed. The chemical shift data, along with that of TC HCl, is presented in Table 16 . The three obscured signals were observed in a spectrum run in protonated Dmso with D_2O capillary to provide lock signal.

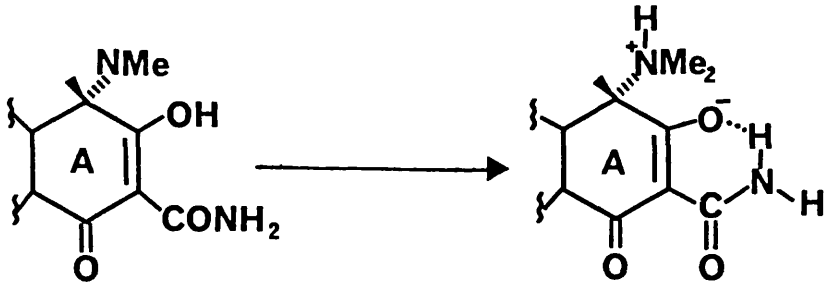


(58) TC base

Table 16 ^{13}C Chemical shift data of TC base and HCl in Dmso.d_6/TMS Underlined resonances are obscured by the solvent multiplet. (TMS = 0 ppm)

Position	TC HCl	TC Base
1	192.9	192.3
2	95.7	98.5
3	187.2	191.8
4	67.9	69.8
4a	35.3	37.4
5	26.9	22.5
5a	42.0	40.8
6	67.9	68.1
6a	147.9	148.0
7	116.9	116.9
8	136.5	136.4
9	115.2	114.5
10	161.3	161.4
10a	114.4	114.4
11	193.4	192.9
11a	106.8	105.9
12	175.0	176.8
12a	43.1	74.3
$CONH_2$	172.1	172.4
C_4-NMe	42.0	42.4
C_6-Me	22.5	22.8

Although assignment of the NMe_2 signal in base and hydrochloride is not entirely unambiguous (both signals occur close to that of C_{5a}), it is clear from data in Table 16 that the NMe_2 resonance undergoes no significant downfield shift when the salt is converted to the free base. Deprotonation of nitrogen would be expected to shift attached methyl carbon resonance downfield (Stothers,1972) therefore it may be concluded that the 4-amino group remains protonated in the free base i.e. the tetracycline molecule exists predominantly in the form of a zwitter-ion. Since ring A has been identified as the first ionisation centre (Knox and Jurand, 1979), loss of C_3-OH proton is most probable as shown (59)

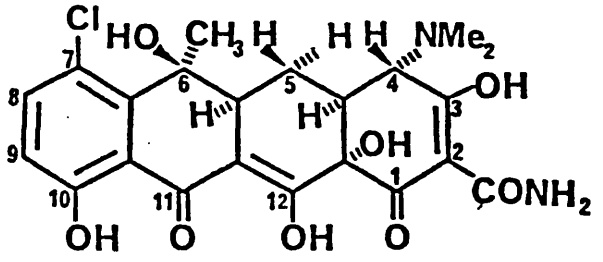


(59)

Coupled with this evidence is the downfield shift (+4.6 ppm) of the C_3 resonance, since β -deshielding by a phenolic function is enhanced after ionization (Stothers, 1972). Small down field shifts at C_2, C_4, C_{4a} and upfield shifts at C_5, C_{12a} support this interpretation.

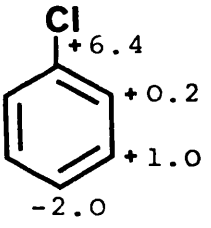
CHLORTETRACYCLINE HYDROCHLORIDE

The spectrum of CTC HCl was run in Dmso.d_6/TMS. A total of 19 signals was clearly resolved plus two obscured by the Dmso.d_6 heptet. The chemical shift values are listed in Table 17.



(1) CTC HCl

The only difference between CTC HCl and TC HCl is the presence of a chlorine substituent at the C_7 position. Accordingly the C_7 doublet in the OFR spcetrum of TC HCl at 116.9 ppm is replaced by a singlet at 121.1 ppm. A downfield shift is expected due to the α -deshielding influence of chlorine (60).



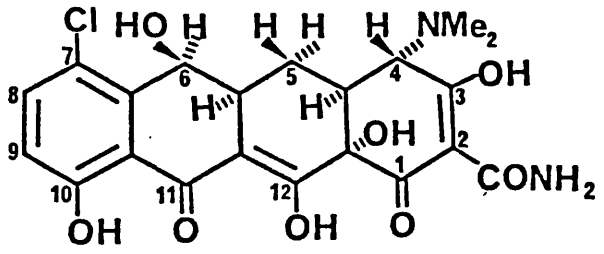
(60) shielding parameters of Cl (Stothers 1972)
(+ deshielded, - shielded)

Table 17. ^{13}C chemical shift data of CTC HCl and TC HCl in Dmso.d_6/TMS. Underlined resonances are obscured by the solvent multiplet. (TMS = 0 ppm)

Position	TC HCl	CTC HCl
1	192.9	192.0
2	95.7	95.8
3	187.2	187.3
4	67.9	68.9
4a	<u>35.3</u>	<u>35.9</u>
5	26.9	27.6
5a	<u>42.0</u>	<u>42.0</u>
6	67.9	70.5
6a	147.9	143.6
7	116.9	121.1
8	136.5	139.7
9	115.2	120.6
10	161.3	160.7
10a	114.4	117.0
11	193.4	193.3
11a	106.8	106.1
12	175.0	175.8
12a	73.1	73.3
$CONH_2$	172.1	172.1
C_4-NMe	<u>42.0</u>	<u>42.8</u>
C_6-Me	22.5	25.1

Resonances in the vicinity of C_7 are also affected by chlorination in approximate agreement with the shielding parameters of chlorine in aromatic systems ((60) and Table 17). One result of this is the resolution of C_4 and C_6 which overlapped in the spectrum of TC HCl.

6-DEMETHYL CHLORTETRACYCLINE HYDROCHLORIDE (6-DEMETHYL CTC HCL)



(4) 6-demethyl CTC HCl

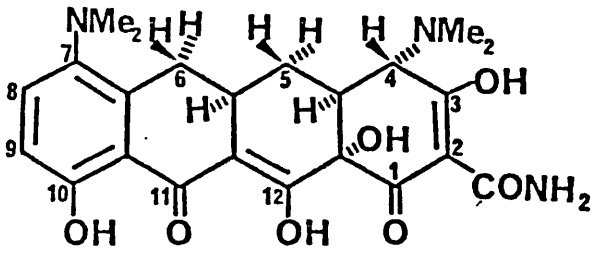
This derivative differs from CTC solely in the absence of a methyl substituent at C_6, so that only two resonances appear upfield of the solvent band. The off-resonance quartet, due to C_6-methyl is absent, and as a result, the C_6 resonance (an OFR doublet) shifts upfield (by 5.7 ppm). The small downfield shifts of C_5 and C_7 may also be explained in terms of the loss of shielding influence of C_6-methyl. The chemical shifts are listed in Table 18.

Table 18. ^{13}C chemical shift data of TC HCl and 6-demethyl CTC HCl in Dmso.d_6/TMS. Underlined resonances are obscured by the solvent multiplet. (TMS = 0 ppm)

Position	TC HCl	6-demethyl CTC HCl
1	192.9	191.6
2	95.7	95.7
3	187.2	187.2
4	67.9	67.9
4a	35.3	35.1
5	26.9	28.2
5a	42.0	36.6
6	67.9	64.8
6a	147.9	140.6
7	116.9	122.1
8	136.5	136.9
9	115.2	118.9
10	161.3	160.0
10a	114.4	115.8
11	193.4	193.3
11a	106.8	105.1
12	175.0	176.1
12a	73.1	73.7
$CONH_2$	172.1	171.9
C_4-NMe	42.0	42.0
C_6-Me	22.5	----

MINOCYCLINE HYDROCHLORIDE

Apart from the dimethylamino substituent at C_7, minocycline (9) represents a further simplification of TC HCl in that it lacks both methyl and hydroxy groups at C_6.



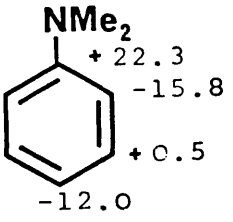
(9) Minocycline

The spectrum of minocycline HCl was run in Dmso.d_6/TMS and showed sixteen well resolved signals to lowfield and four broad signals to higher field plus one signal masked by the solvent multiplet.

Of the highfield signals, C_4 is readilly assigned to 66.7 ppm (doublet in the coupled spectrum) since it is well removed from C_6 and C_7 and is therefore unlikely to be affected by structural variation at these sites. In contrast to an earlier report (Mazzola et al. 1980), resonances at 44.4 and 41.2 ppm are assigned to C_7 and C_4-NMe_2 carbons respectively on the basis of the protonated state of the more basic C_4-NMe_2 group since the α-N-pronation shift is negative (i.e. produces shift to higher field) (Jones and Hassan,

1972). The C_6 resonance of TC HCl suffers a pronounced upfield shift (about 36 ppm) as a result of the absence of C_6 substituent, which also accounts for the shifts of C_{5a} (upfield) and C_5 (downfield).

Two striking shifts of the TC HCl resonances, following insertion of the C_7-NMe_2 group, are the upfield shift of C_{10} and the downfield shift of C_7 (see Table 19). Both are consequences of the shielding influence of NMe_2 upon aromatic carbons, as learnt from data on the model (61).



(61) Shifts relative to those in benzene

Analysis of the four highest field resonances is difficult because their close placing leads to overlap of the coupled spectra. The INEPT programme is, however, well suited to this problem. It is clear from the INEPT spectrum (Fig. 12 ; upfield region only), that resonances at 34.3 and 31.5 ppm are due to methine, and those at 33.7 and 29.6 ppm to be due to methylene (CH_2) carbons. Moreover, the solvent band intensity is very much reduced revealing the two NMe_2 resonances.

Table 19. ^{13}C chemical shift data of TC HCl and minocycline in Dmso.d_6/TMS. Underlined resonances are obscured by the solvent multiplet. (TMS = 0 ppm)

Position	TC HCl	Minocin
1	192.9	192.9
2	95.7	95.6
3	187.2	187.4
4	67.9	68.0
4a	35.3	34.3
5	26.9	29.6
5a	42.0	31.5
6	67.9	33.7
6a	147.9	142.0
7	116.9	136.5
8	136.5	128.5
9	115.2	114.8
10	161.3	157.4
10a	114.4	116.0
11	193.4	193.6
11a	106.8	108.2
12	175.0	174.3
12a	73.1	73.9
$CONH_2$	172.1	171.8
C_4-NMe	42.0	41.2
C_6-Me	22.5	----
C_7-NMe	----	44.4

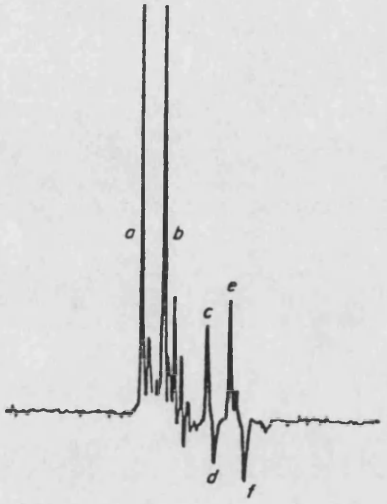


Fig. 12 INEPT ^{13}C NMR spectrum of minocycline HCl in Dmso.d_6 (0-50 ppm region, CH, CH_3 signals positive, CH_2 signals negative). Assignments: a 44.4 ppm C_7-NMe_2; b 41.2 ppm C_4-NMe_2; c 34.3 ppm C_{4a}(CH); d 33.7 ppm C_6(CH_2); e 31.5 ppm C_{5a}(CH); f 29.6 ppm C_5(CH_2).

The compounds discussed up till now i.e. TC HCl, TC base, 6-demethyl CTC, CTC HCl and minocycline, all have one important common structural feature. It is the presence of two hydrogens at C_5 giving rise to a triplet for the carbon resonance in the coupled spectrum. In contrast, OTC HCl, methacycline, meclocycline and doxycycline all have a hydroxy substituent at C_5. This structural feature allows the separation of the tetracycline family into two major groups, with TC and OTC being the basic members.

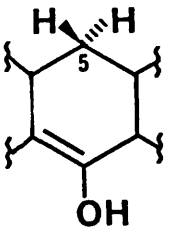
TC HCl

TC base

CTC HCl

DMCTC HCl

Minocycline

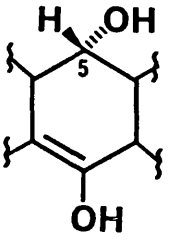


OTC HCl

Methacycline

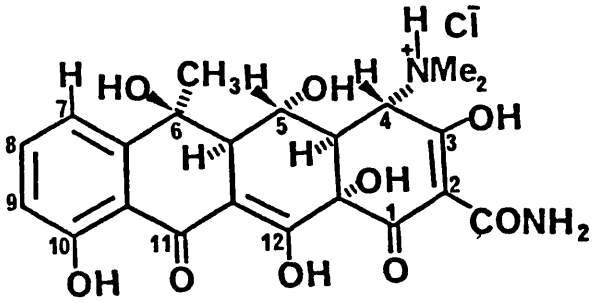
Meclocycline

Doxycycline



As TC HCl has already been discussed in detail, the assignment of OTC HCl will be reported briefly and by comparison with TC HCl.

OXYTETRACYCLINE HYDROCHLORIDE (OTC HCl)

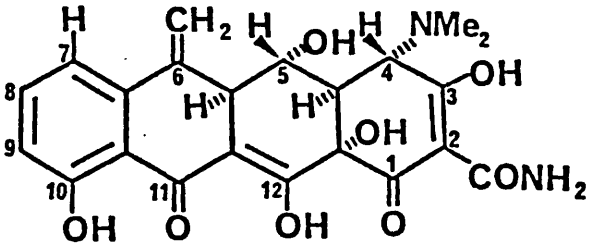


(2) OTC HCl

The spectrum of OTC HCl, together with methacycline, meclocycline and doxycycline, lack the C_5-methylene resonance near 27 ppm (OFR triplet), present in all the spectra discussed so far. Instead, these spectra show a lower field doublet (near 63 ppm), a change due to the common C_5-OH substituent of the group. In this series the

C_{4a} and C_{5a} signals also show downfield shifts relative to the corresponding chemical shifts in TC. The rest of the resonances, presented in Table 20 , have almost identical chemical shifts to those of TC HCl. The broad nature and low intensity appearence of the C_7, C_8 and C_9 and other protonated carbons in Dmso.d_6 spectra, as noted in the case of TC HCl, are also apparent. The T_1 results are discussed on page 108.

METHACYCLINE HYDROCHLORIDE



(5) methacycline HCl

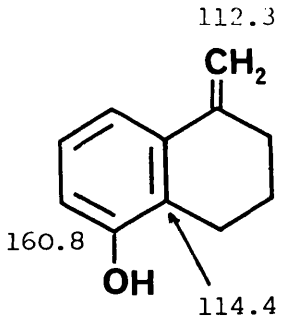
Methacycline differs from OTC in having a methylene function attached to C_6. Its spectrum, along with that of meclocycline (see later) is notable amongst the rest of the group in having no signals upfield of the solvent band. Models are needed to confirm assignments at C_6 since a $=CH_2$ group is not present in compounds discussed up till now.

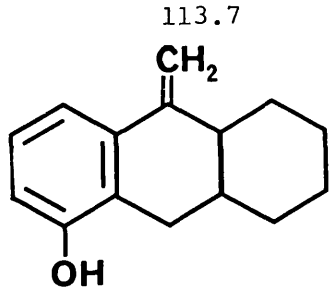
Chemical shift data on models (62) and (63) indicate that the

Table 20. ^{13}C chemical shift data of TC HCl and OTC HCl in Dmso.d_6/ TMS. Underlined resonances are masked by the solvent multiplet. (TMS = 0 ppm)

Position	TC HCl	OTC HCl
1	192.9	192.9,
2	95.7	95.4
3	187.2	187.1
4	67.9	64.9
4a	35.3	<u>42.0</u>
5	26.9	63.2
5a	<u>42.0</u>	49.8
6	67.9	68.9
6a	147.9	148.7
7	116.9	118.9
8	136.5	136.6
9	115.2	114.9
10	161.3	161.2
10a	114.4	114.4
11	193.4	193.7
11a	106.8	105.3
12	175.0	173.5
12a	73.1	72.5
$CONH_2$	172.1	171.9
C_4-NMe	<u>42.0</u>	<u>41.8</u>
C_6-Me	22.5	24.6

vinylic methylene carbon and C_{10a} resonances would be very close. Therefore, the two resonances at 114.4 ppm (singlet in the coupled





(62) Stothers, 1972 (63)

spectrum) and 113.7 ppm (triplet) are assigned to C_{10a} and $C_6{=}CH_2$ respectively. The resonance at 160.7 ppm is assigned to C_{10} by comparison with model (62) and also because of similarity in other tetracyclines. The C_6 signal of OTC moves downfield by about 70 ppm following the change from sp^3 to sp^2 hydridization, while C_{6a} (no longer subject to β -deshielding by oxygen) moves upfield. The other resonances have similar chemical shift values to those of OTC HCl. The chemical shift data is presented in Table 21 along with that of meclocycline.

MECLOCYCLINE

The spectral data, for the base and sulphosalicylate salt, (in

Table 21 . ^{13}C chemical shift data of OTC HCl, methacycline and meclocycline (base and sulphosalicylate salt) in Dmso.d_6/TMS. Underlined resonances are obscured by the solvent multiplet. (TMS = 0 ppm)

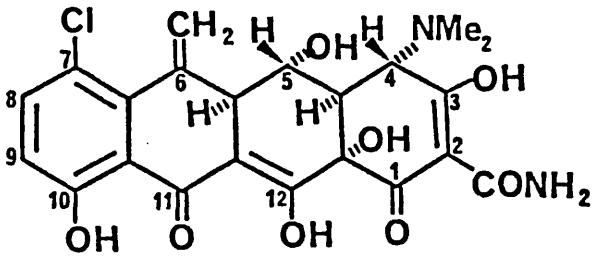
Position	OTC HCl	Methacycline	meclocycline base *	meclocycline salt **
1	192.9	192.1	190.3	190.8
2	95.4	95.0	98.8	94.9
3	187.1	187.3	187.1	186.2
4	64.9	65.3	64.9	63.9
4a	42.0	42.0	40.0	40.0
5	63.2	64.1	65.5	64.4
5a	49.8	44.2	45.4	45.1
6	68.9	140.6	136.9	135.9
6a	148.7	142.6	138.9	138.2
7	118.9	117.3	119.6	119.4
8	136.6	137.0	137.8	138.7
9	114.9	116.4	118.3	118.5
10	161.2	160.7	159.8	158.9
10a	114.4	114.4	116.6	116.6
11	193.7	193.5	191.3	193.2
11a	105.3	105.2	105.4	106.2
12	173.5	173.6	178.2	173.9
12a	72.5	73.5	75.4	73.8
$CONH_2$	171.9	171.6	170.5	171.0
C_4-NMe	41.8	42.0	41.7	41.0
C_6-Me	24.6	----	----	----
C_5=CH_2	----	113.7	117.4	117.5

* Mazzola et al. 1980

** the resonances due to the organic salt are at 116.6, 126.9, 132.6, 136.0, 137.7 and 160.4 ppm.

Dmso.d_6/TMS) are presented in Table 21.

The ^{13}C NMR data on the meclocycline base has already been published (Mazzola et al. 1980). During the present work, the sulphosalicylate salt was studied.

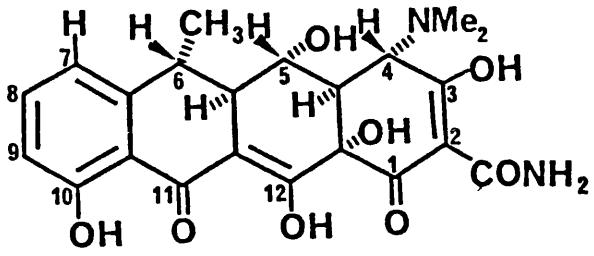


(64) Meclocycline

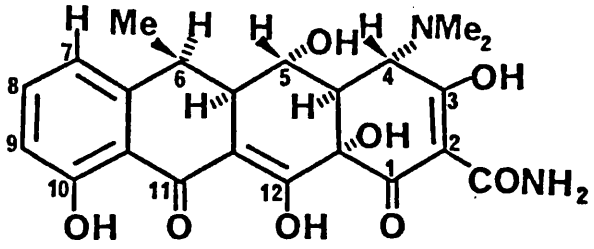
Meclocycline differs from methacycline by the presence of a substituted chlorine at C_7. The ^{13}C spectrum does not show any resonances upfield of the solvent multiplet (similar to that of methacycline). A downfield shift is expected at C_7 due to the deshielding effect of Cl, but the actual shift is very small. The rest of the chemical shifts are very similar to the base, and they correlate well with those of methacycline hydrochloride.

6-DEOXY OTC (DOXYCYCLINE) AND ITS 6 β-METHYL ISOMER

The official composition of doxycycline hydrochloride (6)



(6) doxycycline



(65) 6-epi doxycycline

(Martindale, 1980) is as follows:-

Doxycycline hyclate (U.S.P.):- Doxycycline hemi-ethanolate hydrochloride hemi-hydrate

i.e. the hydrochloride salt crystallizes with half a mole each of water and ethanol.

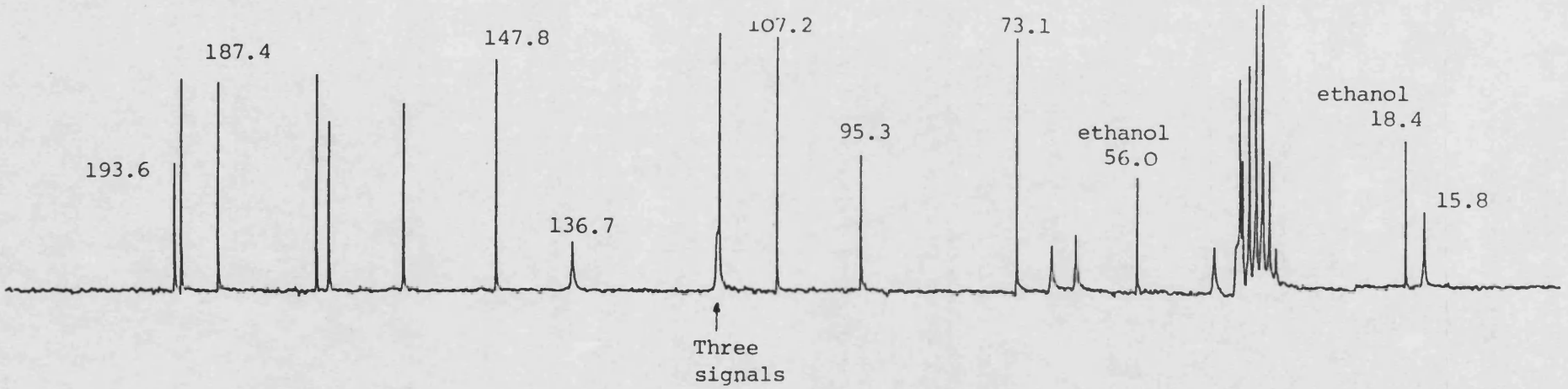
Therefore two ethanol peaks must also be accounted for. The ^{13}C spectrum of doxycycline displayed 23 resonances (21+2 for ethanol). The resonances due to ethanol of crystallisation are 18.4 ppm (OFR quartet due to CH_3) and 56.0 ppm (OFR triplet due to CH_2).

The absence of a C_6-OH substituent is reflected in the upfield shifts of C_6 and neighbouring carbon resonances, most pronounced for C_6(-28 ppm), moderate for C_6-methyl (-8.8 ppm) and C_{5a} (-4.6 ppm) by comparison with the corresponding shifts of OTC. The near coincidence of the C_7, C_9 and C_{10a} resonances is a notable feature of the spectrum, as shown in Spectrum 14 . The T_1 data is discussed on page 111.

The C_6 epimer of doxycycline (65) is a possible impurity since catalytic reduction of its precursor (methacycline) is not stereospecific (Stephen et al. 1963).

The spectrum of the C_6 epimer of doxycycline differs notably from that of the more active antibiotic in:-

Spectrum 14.



Complete decoupled spectrum (^{13}C NMR) of doxycycline hyclate in Dmso.d_6/TMS.

1) Clear resolution of C_7, C_9 and C_{10a} resonances.

2) Reduced separation of C_4 and C_5 signals ($\Delta\delta$ ppm 0.6 ppm; 3.5 ppm for doxycycline).

3) Resolution of the C_{4a} resonance to highfield of the solvent band.

4) Absence of ethanol peaks.

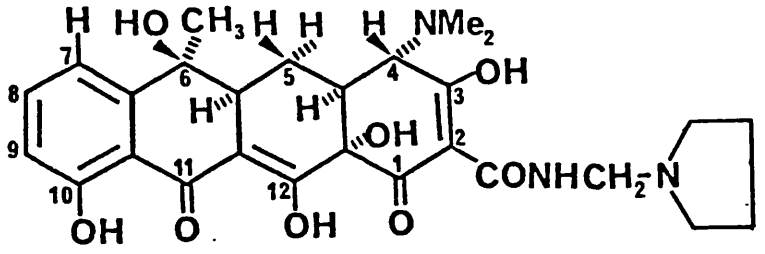
Thus the presence of the C_6-epimer in a doxycycline sample may be readily detected from its ^{13}C NMR spectrum. Furthermore, the absence of such diagnostic signals is evidence of isomeric purity, as was the case for the sample reported here. Most chemical shift differences between corresponding carbons of doxycycline and its epimer are consistent with those expected from replacement of an α by a β-C_6-methyl. For example, upfield shifts of C_{10a} and C_{11a} resonances due to γ-deshielding are observed. The chemical shift data of doxycycline, 6-epi doxycycline and OTC HCl are presented in Table 22.

Table 22. ^{13}C chemical shifts of OTC, doxycycline and 6-epi-doxycycline. Underlined resonances are masked by solvent multiplets. (TMS = 0 ppm)

Position	OTC HCl	Doxycycline	6-epi doxycycline
1	192.9	192.6	192.5
2	95.4	95.2	95.0
3	187.1	187.4	187.2
4	64.9	68.1	64.7
4a	42.0	41.0	32.9
5	63.2	64.6	63.8
5a	49.8	45.2	43.3
6	68.9	41.0	41.2
6a	148.7	147.7	150.0
7	118.9	115.5	118.7
8	136.6	136.7	137.3
9	114.9	115.5	115.6
10	161.2	161.1	161.3
10a	114.4	115.4	114.3
11	193.7	193.6	193.7
11a	105.3	107.2	103.5
12	173.5	173.5	174.8
12a	72.5	73.0	73.8
$CONH_2$	171.9	171.7	171.6
C_4-NMe	41.8	41.0	41.2
C_6-Me	24.6	15.8	16.6

SEMI SYNTHETIC TETRACYCLINES

Rolitetracycline (41) and lymecycline are both Mannich bases formed from tetracycline, formaldehyde and pyrrolidine or lysine. The spectrum of rolitetracycline is very similar to that of TC base, but has the distinguishing feature of additional signals at 56.6, 50.1 and 23.6 ppm due to pyrrolidino-methylene unit (Mazzola et al. 1980).



(41) rolitetracycline

An attempt to record the spectrum of lymecycline in D_2O (it is insoluble in Dmso.d_6) at the usual concentration (50-60 mg in 0.5 ml solvent) failed because of the rapid formation of a precipitate from the initially clear solution. The spectrum of the supernatant liquid displayed six resonances due to lysine (176.0, 55.9, 40.5, 31.3 and 22.7 ppm (Rabenstein and Sayer, 1976) plus one extra resonance (35.9 ppm) probably due to the hydrated formaldehyde. However the spectrum

of the precipitate in Dmso.d_6 showed the material to be TC base. It proved possible to record a spectrum of a more dilute solution (4mg in 0.5ml D_2O) at higher frequency (100 MHz), which displayed all the lysine and many of the TC signals. It is evident that lymecycline is a highly unstable complex that rapidly reverts to its component parts on dissolution. The chemical shift data of rolitetracycline is presented in Table 23..

Table 23. ^{13}C Chemical shift data of TC base and rolitetracycline.

Underlined resonances are obscured by the solvent multiplet.(TMS = 0 ppm)

Position	TC HCl	rolitetracycline*
1	192.9	191.8
2	95.7	101.2
3	187.2	189.6
4	67.9	72.5
4a	35.3	38.6
5	26.9	22.5
5a	<u>42.0</u>	<u>41.6</u>
6	67.9	68.0
6a	147.9	148.0
7	116.9	116.7
8	136.5	135.9
9	115.2	115.1
10	161.3	161.4
10a	114.4	114.5
11	193.4	192.0
11a	106.8	105.3
12	175.0	180.1
12a	<u>43.1</u>	<u>74.8</u>
$CONH_2$	172.1	168.3
C_4-NMe	42.0	42.3
C_6-Me	22.5	22.9

* additional signals: 23.6 and 50.1 ppm pyyrolidine carbons, 56.6 ppm CH_2N. (Mazzola et al. 1980)

CHAPTER 4

SPECTRAL ANALYSIS OF THE DEGRADATION PRODUCTS OF TETRACYCLINE GROUP OF ANTIBIOTICS

Section 1

^{1}H NMR

4-epi TC HCl

4-epi CTC HCl

Anhydro TC HCl

Anhydro CTC HCl

Iso CTC base

^{1}H NMR

4-EPI TC HCl (12)

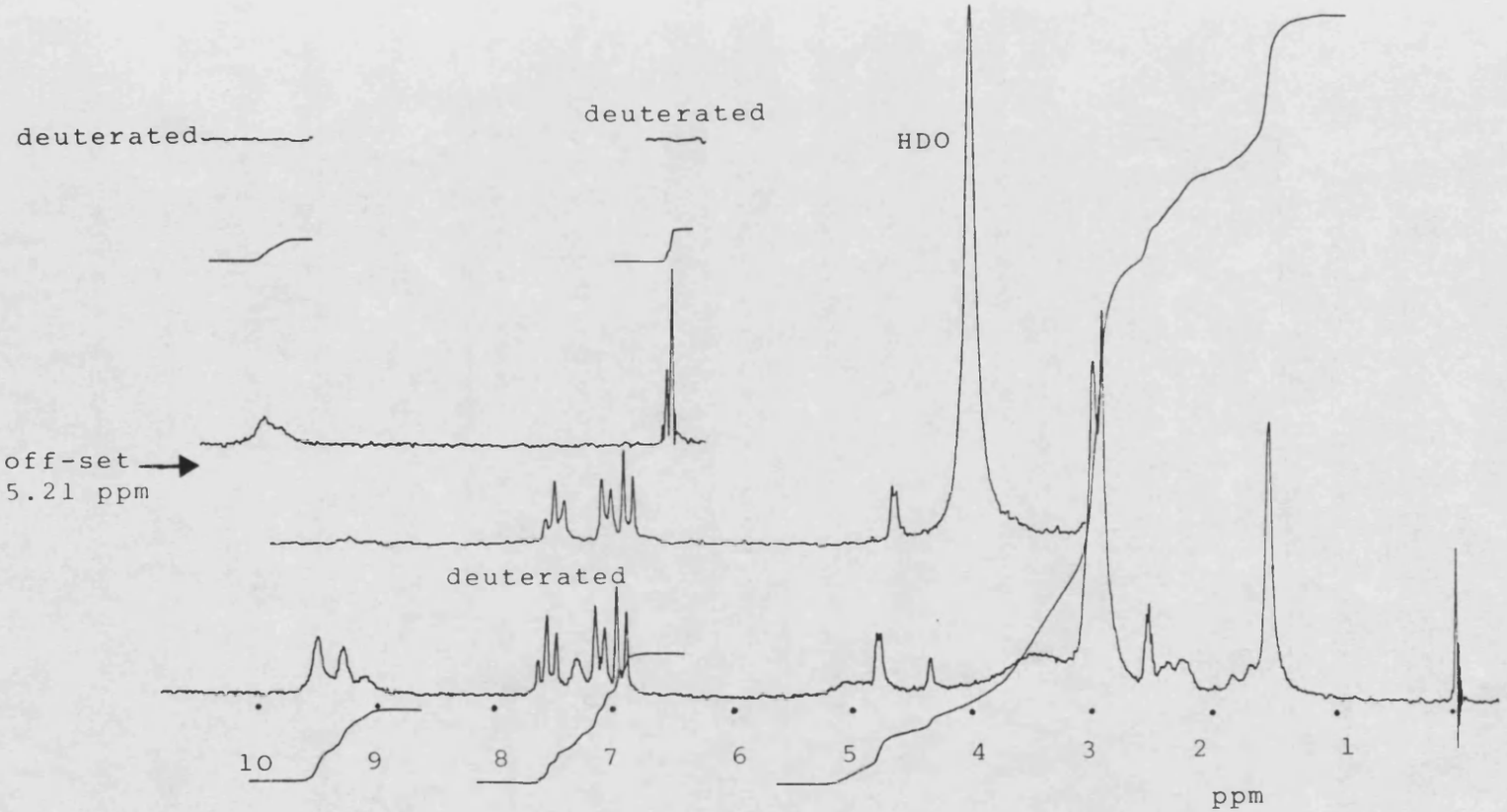
The spectra of most TC HCl samples displayed a low intensity broad band or narrow doublet to lowfield (~ 4.8 ppm) of the 4-H resonance (~ 4.3 ppm) which is indicative of the presence of 4-epi TC as an impurity. A commercial sample of 4-epi TC HCl, obtained from Pfizer laboratories, gave a spectrum which likewise showed resonances at 4.75 ppm (narrow doublet, separation 3-4 Hz) and 4.3 ppm (broad singlet) , but with the lowfield signal of greater intensity. This sample was evidently a mixture of the two isomers. A sample run on an HPLC column invariably showed an epi TC peak appearing as a shoulder on the ascending part of the main TC peak. The mobile phase had to be modified to separate the TC and 4-epi TC peaks (see Chapter 6).

The only other ^{1}H NMR spectral difference of significance was the fact that the NMe_2 resonances of the 4-epi derivative was broader than that of the TC HCl (see also results for CTC and 4 epi-CTC). In the spectra of mixtures closely placed broad and narrow singlets were seen near 3 ppm (Spectrum 15). Chemical shifts in Table 24.

4-EPI CTC HCl

This sample was derived from Lederle laboratories which appeared fairly pure from its ^{13}C NMR spectrum .

Spectrum 15. ^{1}H NMR spectrum of 4-epi TC HCl in Dmso.d_6/TMS run at 100 MHz.



The material provided a well resolved 400 MHz ^1H spectrum and this allows spectral differences between 4-epimer TC derivatives to be well defined. The comparative analysis of spectra of CTC HCl and its 4-epi isomer, recorded under the same conditions (400 MHz in Dmso.d_6) is shown below:

Compound	C-6 methyl	C-4 NMe_2	H-4	H-4a	H-5α and/or H-5β	H-5a
Chlortetracycline HCl* [(2)HCl]	1.84 s	2.87 bs	4.32 bs	2.92 bd (13)	α: 2.24 ddd (2, 5, 13) β: 1.71 bq (12)[d]	2.99 dd (5, 10.5)
4-*epi*-Chlortetracycline HCl* [(3)HCl]	1.80 s	Very broad band beneath H-4a and H-5a signals	4.74 d (3.80)	2.91 dt (4.4, 14)	α: 2.17 bd (14) β: 1.58 bq (10.5)[d]	3.00 dd (5, 11)

Chemical shifts in ppm from TMS, coupling constants (Hz) in parentheses following chemical shift. Abbreviations: b=broad, s=singlet, d=doublet, t=triplet, q=quartet,

Note:

1) The 4-H resonance of the 4-epi isomer (4.74 ppm, separation 3.8 Hz) is downfield of the corresponding 4-H signal of CTC (4.32 ppm, broad singlet).

2) The pronounced broadness of the NMe_2 resonance of the 4-epi isomer compared with that of CTC (see Fig. 4 page 74).

3) The 4a-H resonance of 4-epi CTC shows an addational coupling compared with the multiplication of the 4a-H signal of CTC due to the significant coupling with 4-H (~4 Hz). The conformation of CTC and 4-epi CTC has already been discussed (see Chapter 2).

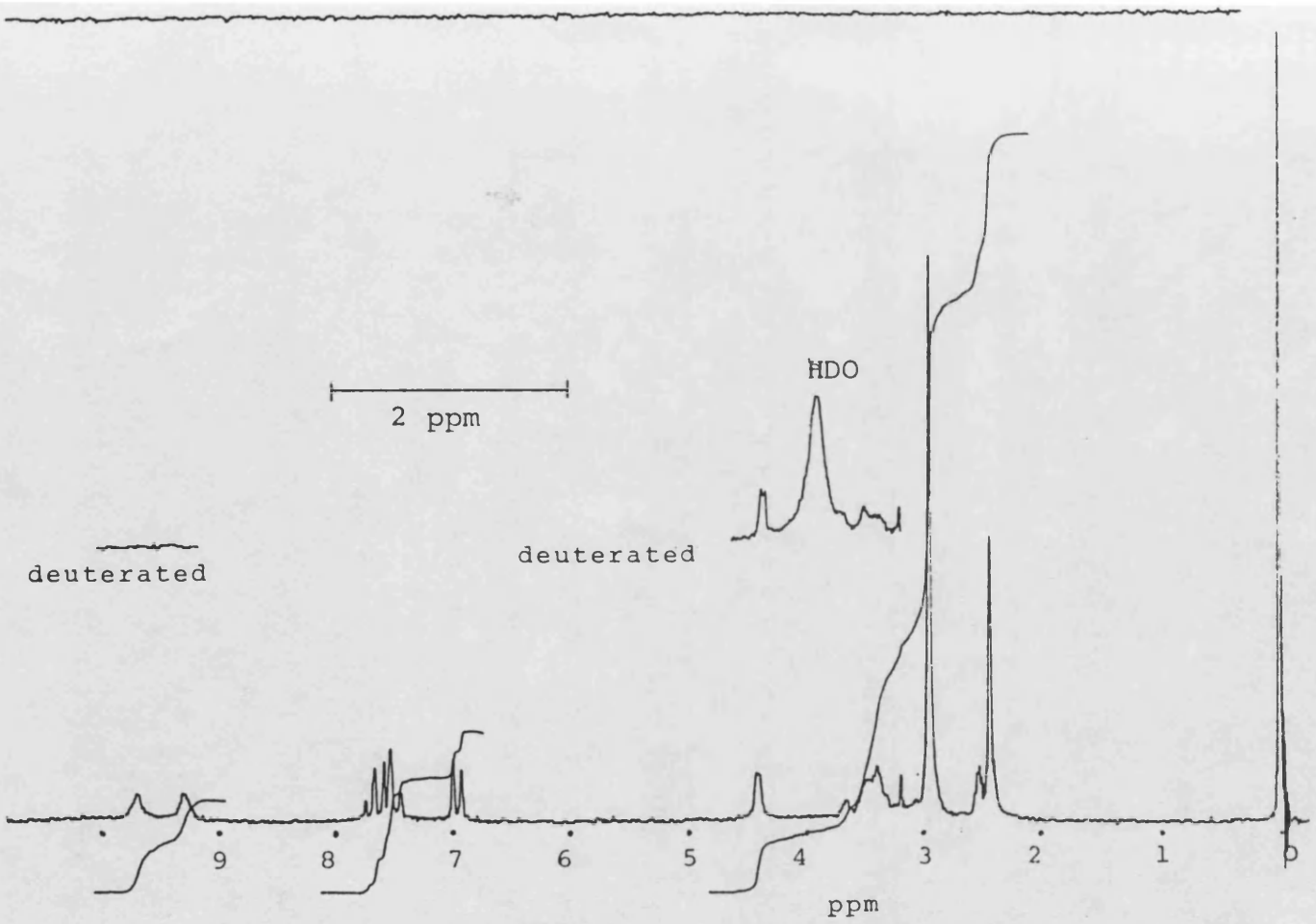
ANHYDRO TC HCl

Anhydro TC HCl was prepared in the laboratory as discussed on page 40. A ^1H NMR spectrum was run at 100 MHz in Dmso.d_6 (Spectrum 16).

The C_6-methyl forms a sharp resonance near 2.5 ppm. The downfield shift of this signal (cf. TC HCl 1.5 ppm), as a result of the change of the C_6 carbon from sp^3 to sp^2 hybridization yields a significant difference between the spectra of two compounds. This may be used to monitor the dehydration of the parent compound. The chemical shifts of 4-H and C_4-NMe_2 were unaffected (due to remoteness of these protons from the site of dehydration). The rest of the methine signals were unresolved within the 1.6-3.2 ppm envelope. (Table 24).

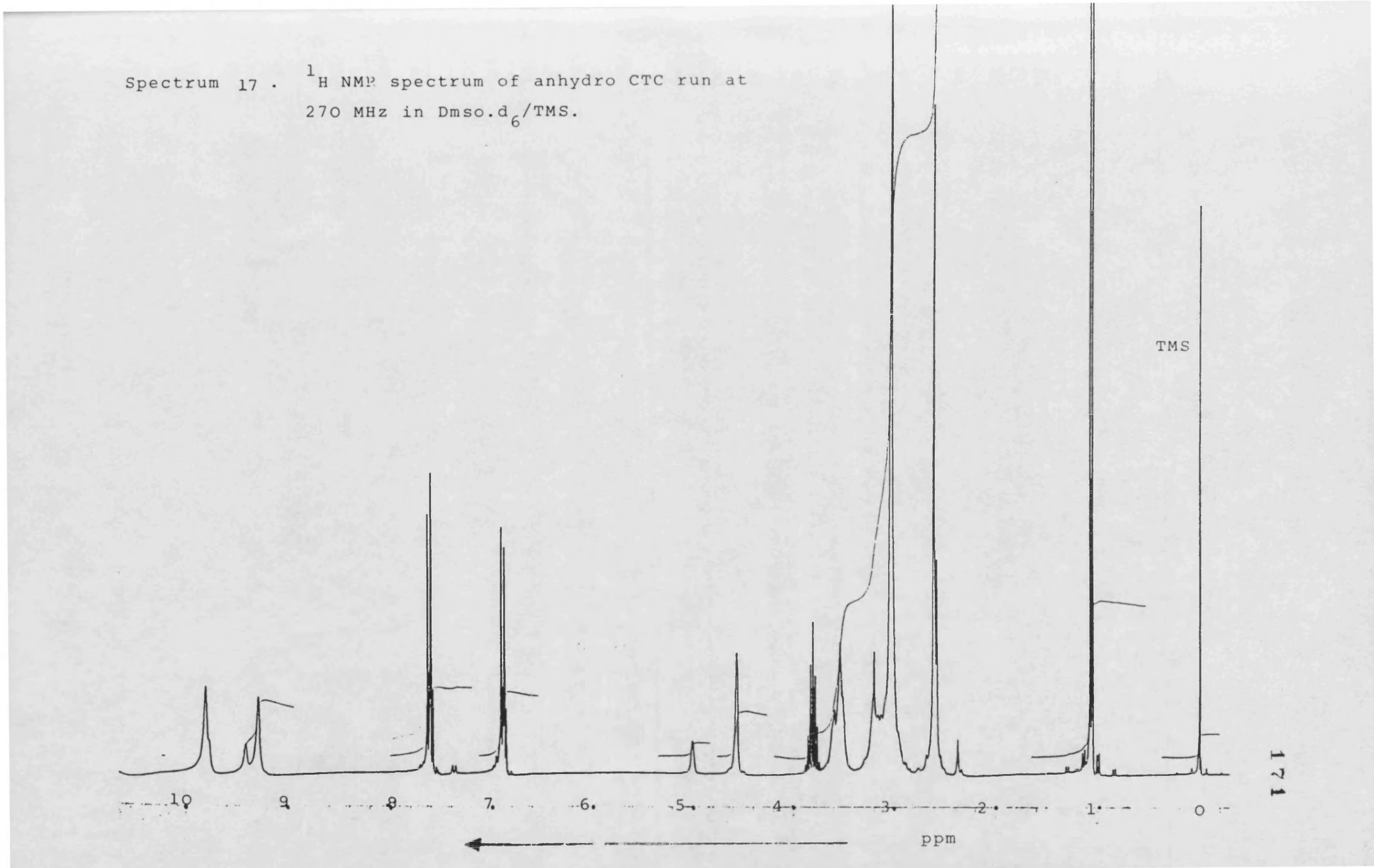
ANHYDRO CTC HCl

Anhydro CTC HCl was prepared in the laboratory following the same procedure as that used for anhydro TC. A ^1H NMR spectrum was run at 270 MHz in Dmso.d_6/TMS (Spectrum 17). (Table 24)



Spectrum 16 . ^{1}H NMR spectrum of anhydro TC HCl at 100 MHz in Dmso.d_6/TMS.

Spectrum 17 . ^{1}H NMR spectrum of anhydro CTC run at 270 MHz in Dmso.d_6/TMS.



The resonances near 1 and 3.8 ppm are due to residual iso-propanol, used in the dehydration procedure.

Due to the presence of C_7 substituted chlorine, the normal aromatic pattern of one triplet and two doublets (e.g. as in TC HCl) is altered to a pair of doublets.

The anhydro CTC spectrum is very similar to that of anhydro TC. The C_6-methyl resonance displayed the expected downfield shift due to deshielding influence of chlorine (anhydro TC 2.4; anhydro CTC 2.6 ppm). The spectrum of anhydro CTC was characterised by the C_6-methyl signal (2.6 ppm), 4-H signal (4.6 ppm) and A B aromatic 4-line resonance in accordance with the same features of anhydro TC after allowance for the influence of the 7-chloro substituent. All these signals were accompanied by duplicate resonances of lower intensity (See Spectrum 18), evidence that the 4-epi anhydro CTC isomer be present.

ISO-CHLORTETRACYCLINE (ISO-CTC)

In alkaline solutions, tetracyclines having a C_6-OH group degrade readily to the iso-tetracycline derivative (as described on page 18). CTC is specially prone to this type of degradation.(Stephen et al. 1954).

^1H

The ^1H NMR spectrum was run at 270 MHz in Dmso.d_6/TMS. The spectrum was complex and displayed duplicate aromatic and C_6-methyl signals indicating the presence of an epimeric mixture.

Spectrum 18 . Expanded spectrum of anhydro CTC (270 MHz in Dmso.d_6/TMS.)

Table 24. Chemicla shifts (^{1}H NMR) of TC HCl, 4-epi TC HCl, anhydro TC and anhydro CTC HCl recorded at 100 MHz in Dmso.d_6/TMS

		TC HCl	4-epi TC HCl	Anhydro-TC HCl	Anhydro-CTC HCl
Ring D aromatics		7.5 t, 7.1, 6.9 d	7.5 t, 7.1, 6.9 d	7.6 m, 6.9 d	7.6 m 6.9 d
C_6methyl		1.5 bs	1.5 bs	2.4 bs	2.6 bs
C_4NMe_2		2.9 bs	2.9 s 3.0 bs	2.9 s	3.0
ACIDIC	$CONH_2$	9.5, 9.1	9.5, 9.3	9.7, 9.3	9.3, 9.8
	OTHERS	11.7, 15.0	11.7, 15.1		
METHINE	C_4	4.3 bs	4.75, 4.3 bs	4.4	4.6
	C_5	unresolved	unresolved	unresolved	unresolved
	C_{4a}	"	"	"	"
	C_{5a}	"	"	"	"

s singlet, d doublet, t triplet, m multiplet

SPECTRAL ANALYSIS OF DEGRADATION PRODUCTS OF TETRACYCLINES

Section 2

^{13}C NMR

4-epi TC HCl

Anhydrotetracycline HCl

^{13}C NMR assignments of resonances

Anhydro CTC HCl

α and β -apo oxytetracycline bases

^{13}C NMR assignments

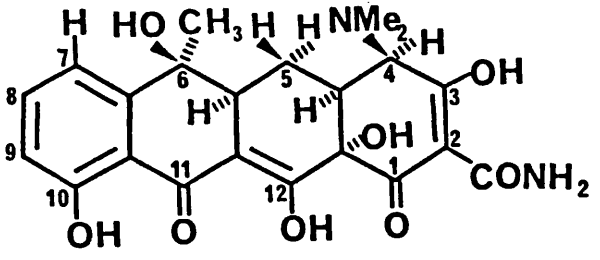
Iso CTC base

SPECTRAL ANALYSIS OF DEGRADATION PRODUCTS OF TETRACYCLINE GROUP OF ANTIBIOTICS

^{13}C- NMR

4-EPI TC HCl

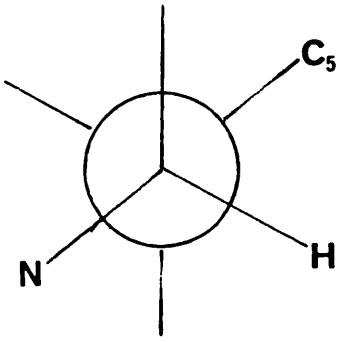
4-epi TC HCl is stereochemically different from TC HCl in respect of its configuration at C_4. In TC, the C_4NMe_2 substituent lies in a plane below the mean plane of the tetracycline molecule when depicted in the normal manner (3) as do the C_6-methyl and C_{12a}-OH groups, whereas in 4-epi TC HCl, the C_4-NMe_2 is directed above this plane. In the two dimensional representation, as shown below (12), this geometry is depicted by a heavy line for the C_4-NMe_2 bond (above plane of paper and a dotted line for the C_4-H bond (below plane of paper).



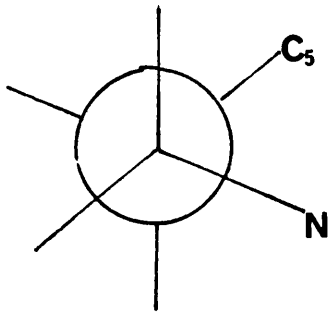
(12) 4-epi TC

Due to this change in stereochemistry at C_4, the following carbon atoms are anticipated to show change of chemical shift: C_3, C_4, C_{4a}, C_4-NMe_2 and C_5. The rest of the carbons are too far removed to be affected to any appreciable extent. Assignments of these latter carbons may thus be made by comparison with the chemical shifts of similar carbons in TC HCl.

In the ^{13}C NMR spectrum of 4-epi TC HCl, all resonances to low field of 65 ppm were similar to the corresponding signals of TC HCl, except that the C_4 and C_6 resonances were resolved in the spectrum of the 4-epi isomer. The C_5 resonance shifts from 26.9 ppm (in TC) to 19.8 ppm in 4-epi TC. This may be explained due to the γ - shielding effect of NMe_2, since its positioning now makes it γ -gauche to C_5 (66).



TC



4-epi TC

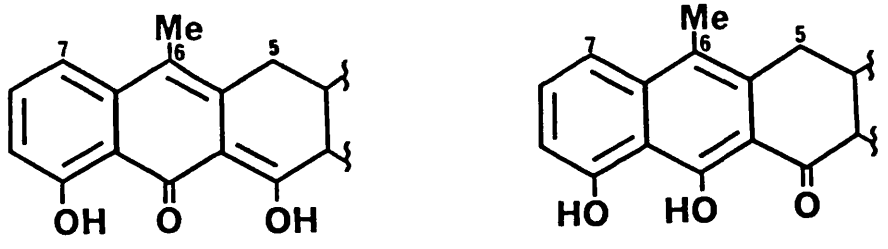
(66)

The C_{4a} resonance, just downfield of the Dmso.d_6 signal, may have

shifted due to the same steric influence. The rest of the resonances have correspondingly similar values. The presence of C_4-epi TC in a sample of TC is therefore best detected by the appearence of a resonance upfield of the 22.5 ppm C_6-methyl signal. The ^{13}C chemical shift data are presented in Table 25.

ANHYDROTETRACYCLINE (anhydro TC HCl)

The mechanism of anhydro formation and the stereochemical reason favouring the dehydration has been discussed (page 15). The anhydro TC may be presented as a tautomeric mixture, but the ^{13}C NMR data indicate structure (14b), with both rings C and D aromatic, to be the principal component as discussed below.



a b

(14) Anhydro tetracycline (partial structure)

^{13}C-NMR ASSIGNMENTS OF RESONANCES

The proton noise decoupled spectrum of anhydro TC HCl displays the

Table 25. ^{13}C chemical shifts of some tetracycline derivatives in Dmso.d_6/TMS. Underlined resonances are masked by the solvent multiplets. (TMS = 0 ppm)

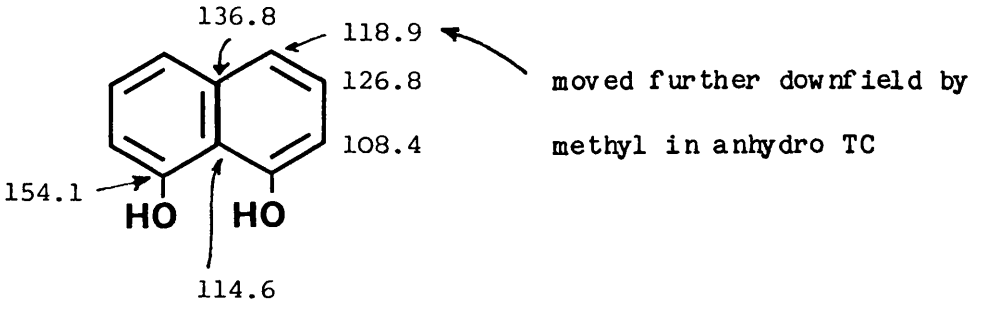
Position	C_4epi TC	Anhydro TC	β-apo OTC	α-apo OTC
1	191.0	192.8	195.6	195.1
2	95.5	97.8	98.3	98.9
3	187.8	187.4	197.6	191.2
4	66.0	66.9	63.5	63.0
4a	43.3	42.0	45.0	34.4
5	19.8	28.7	70.9	69.4
5a	<u>42.0</u>	121.6	143.4	141.6
6	68.0	130.5	114.9	113.4
6a	148.0	138.8	138.4	138.9
7	117.5	114.9	115.3	115.9
8	136.5	132.4	129.6	130.0
9	115.9	111.3	113.9	111.8
10	161.4	157.9	156.3	162.4
10a	114.5	112.1	105.2	102.5
11	193.0	163.6	154.7	159.8
11a	106.6	108.6	108.9	108.9
12	175.1	199.3	168.3	168.2
12a	73.7	76.2	76.0	75.5
$CONH_2$	172.9	172.1	173.1	172.6
C_4NMe_2	<u>42.0</u>	<u>42.0</u>	<u>42.0</u>	<u>42.0</u>
C_6-Me	22.4	14.1	13.8	4.0

required 21 signals (2 to highfield, 17 to lowfield and 2 within the solvent band). In TC HCl, the three lowest field signals were observed at 193.4, 192.9 and 187.2 ppm and assigned to C_{11}, C_1 and C_3 respectively. As C_1 and C_3 are well removed from the structural changes following dehydration their chemical shifts are expected to be similar in the two compounds. Therefore 192.7 and 187.6 ppm resonances are assigned to C_1 and C_3 respectively. Other carbons having similar environments to corresponding carbons of TC (e.g. C_{10} and $CONH_2$) may also be assigned by direct chemical shift comparisons.

In anhydro TC, C_{11} is phenolic in the 14b formulation, therefore an upfield shift is expected, whereas the C_{12} of TC HCl becomes ketonic in the anhydro derivative and would be expected to move downfield. Hence, resonances at 163.6 and 199.3 ppm are assigned to C_{11} (see below) and C_{12} respectively.

The two signals immediately upfield of the $CONH_2$ signal i.e. at 163.6 and 157.9 ppm, have chemicl shifts similar to the C_{10} resonance of TC HCl and are thus linked to C_{10} and C_{11} carbons, both of which are phenolic in anhydro TC. Since C_{11} is closer to the C_{12} carbonyl and does not possess β -hydrogens attached to carbon, the lower field signal is assigned to C_{11}, while the C_{10} shift must be 157.9 ppm (fully coupled spectrum, narrow doublet due to one β C-H)

An upfield shift of the C_{6a} resonance is expected by comparison with that of TC HCl (147.9 ppm) as a result of the absence of β - deshielding by oxygen. Chemical shift data for 1,8-dihydroxy-naphthalene (56) supports a 138.8 ppm assignment for C_{6a}.



(56) 1,8-dihydroxy naphthalene

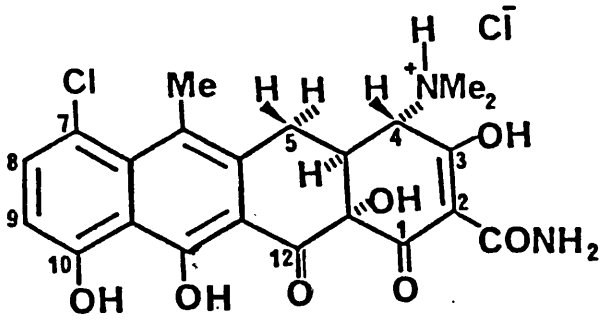
The C_7, C_8 and C_9 resonances may be identified by their broad, low intensity nature; chemical shifts for these carbons are 114.9, 132.4 and 111.3 ppm respectively. A unique feature of the anhydro TC spectrum (^{13}C) is the presence of two resonances (singlets in coupled spectrum) at 130.5 and 121.6 ppm between the C_8 and C_7 signals. The resonance at 130.5 ppm is assigned to C_6 (cf. 56) and that at 121.6 ppm to C_{5a} (both carbons are aliphatic in TC HCl).

The remaining resonances may be all assigned from model data (e.g C_{10a}, 112.1 from (56)) and by direct comparison with the spectrum of TC HCl, since little difference in the environment of

corresponding carbons is involved. Of the higher field resonances of TC HCl, that of C_{4a} moves dowfield into the solvent multiplet, while the C_6-methyl signal moves upfield (absence of β -deshielding oxygen) after dehydration. The ^{13}C chemical shifts are listed in Table 25.

If the tautomeric form (14a) of anhydro TC is significantly populated, we would not expect major changes in the chemical shifts of C_{11} and C_{12} from the corresponding values in TC HCl. In fact C_{11} and C_{12} in particular show radical changes in their chemical shifts due to the structural changes in the anhydro derivative. It is possible that the structure (14a) is formed initially during dehydration but quickly reverts to the more stable form (14b).

ANHYDRO CTC HCL (67)



(67) anhydro CTC HCl

A sample of anhydro CTC HCl was prepared in the laboratory from CTC HCl following the method for anhydro TC HCL (page 40). A ^{13}C NMR spectrum (at 67.8 MHz) was run in Dmso.d_6/TMS. The chemical shifts are presented in Table 26 along with those of anhydro TC HCl.

The only difference between the anhydro derivatives of TC and CTC is the presence of a chlorine substituent at C_7 in anhydro CTC. Accordingly the C_7 doublet in the OFR spectrum of anhydro TC (at 114.9 ppm) is expected to be replaced by a singlet lowerfield of the original TC signal due to the deshielding influence of chlorine atom. (60 on page 143). Resonances in the vicinity of C_7 should also be affected by chlorination.

Since the ^{13}C NMR assignments of anhydro TC HCl have already been discussed, the assignments of CTC HCl will be by comparison with the resonances of the former compound.

C_6-methyl resonance in anhydro CTC shifts lowerfield to 19.1 ppm (cf. anhydro TC 14.1 ppm) due to the deshielding influence of chlorine. Similar influenmce accounts for the C_6 resonance near 136 (cf. anhydro TC 130.5 ppm) and C_7 (anhydro TC 114.9 ppm; anhydro CTC 119.2 ppm). The remaining resonances are very similar to those of anhydro TC.

The ^{13}C NMR spectrum showed the sample to be contaminated either by

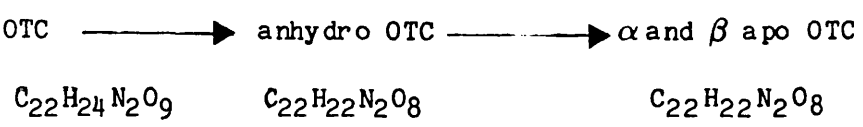
Table 26 . ^{13}C NMR chemical shifts of anhydro TC HCl (22.5 MHz) and anhydro CTC HCl (67.8 MHz) in Dmso.d_6/TMS. Underlined resonances are obscured by the solvent multiplet. (TMS = 0 ppm)

Carbon	anhydro TC HCl	anhydro CTC HCl
12	199.3	199.6
1	192.8	192.6
3	187.4	187.3
$CONH_2$	172.1	172.5
11	163.6	163.1
10	157.9	157.4
6a	138.8	135.6
8	132.4	133.8
6	130.5	136.1
5a	121.6	121.4
7	114.9	119.2
10a	112.1	114.3
9	111.3	111.6
11a	108.6	108.9
2	97.8	97.5
12a	76.2	76.1
4	66.9	66.8
C_4NMe_2	<u>42.0</u>	<u>42.0</u>
4a	<u>42.0</u>	<u>42.0</u>
5	28.7	25.3
C_6-Me	14.1	19.1

impurities or epimeric degradation products. A sample of CTC HCl run on the HPLC column showed the drug to be a mixture of at least two other components as well as the main drug.

α AND β -APO OXYTETRACYCLINE BASES

The first published report on the apo-derivatives of OTC was presented by Hochstein et al. (1953). The authors discovered the following pathway leading to the formation of apo OTC (isolated in the isomeric forms, α and β of unknown stereochemistry) under conditions of low pH.

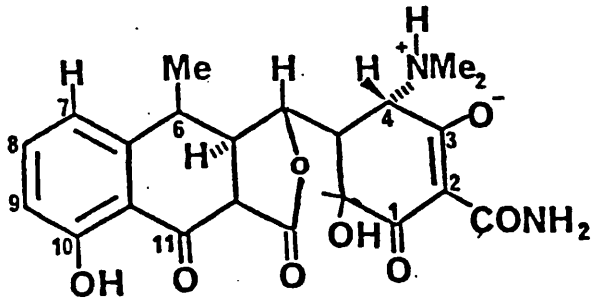


The apo derivatives were prepared and isolated as the free bases as described in Chapter 1.

^{13}C NMR ASSIGNMENTS

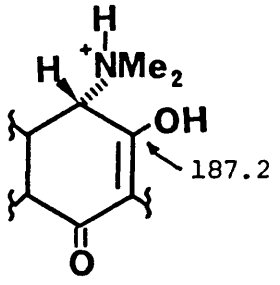
THE ^{13}C NMR spectrum of the β-isomer displayed the required number of resonances (19 to lowfield, 1 to highfield and 1 within the solvent multiplet), confirming that no loss of carbon had occurred during the transformation. Spectral analysis is carried out in

relation to the zwitter ion structure (16), containing a C_1,C_{12a}-ketol unit. The two lowest field singlet resonances (at 197.6 and 195.5 ppm) suggest association with carbonyl carbons. One must be due to C_1, but the second one cannot be due to C_{12} (lactone carbonyl), since the chemical shift is much lower field than expected for such carbons. Assignment of one of these two resonances to C_3 is

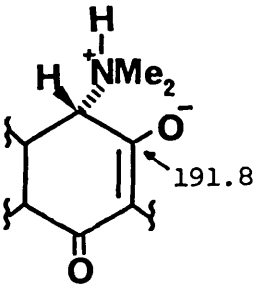


(16) β -apo OTC base

appropriate, however, because an enolic carbon has a chemical shift greater than 190 ppm when ionized, as indicated by data on TC base and HCl already discussed.

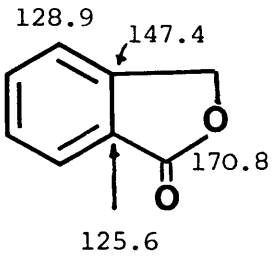


TC HCl

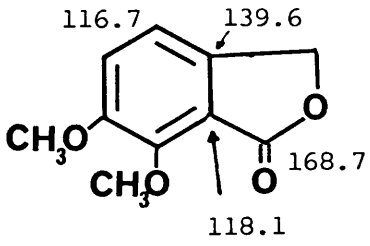


TC base

The next lower-field resonance, at 173.1 ppm, is assigned to $CONH_2$ in accordance with the shift of the corresponding carbon of TC and other TC derivatives. Phthalide (68) and related derivatives (69) provide valuable clues to the lactone carbonyl (C_{12}) and ring C assignments. From these results, the resonance at 168.3 ppm is assigned to the lactone carbon (C_{12}). Resonances at 156.3 and 154.7 ppm are attributed to the phenolic carbons C_{10} and C_{11} (cf shifts of



(68)



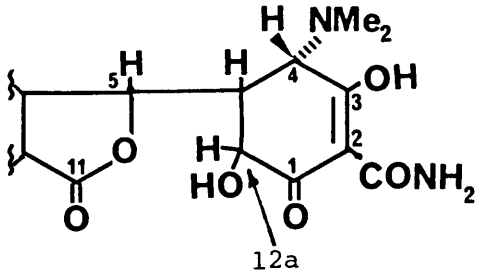
(69)

analogous carbons of anhydrous TC), while the C_7, C_8 and C_9 assignments follow in the usual way from the characteristic signal appearance (decoupled spectrum: low intensity and broad nature; fully coupled spectrum: doublets). Comparison with model data (anhydro TC and (69)) also lead to the assignment of C_{5a} (143.4 ppm) and C_{6a} (138.4 ppm) resonances.

Assignment of the remaining quaternary carbon signals, except that of

C_2 (98.2 ppm) presents some difficulty. Chemical shifts of the three signals still left to be allocated (105.2 ppm and two near 115 ppm) are appropriate for C_{10a} and C_{11a}, but seem too low for C_6 (cf. 130.6 ppm in anhydro TC). But this low field shift may provide a clue to the configuration of the molecule if the stereochemistry about C_5 is such that C_6 is subject to steric polarization by the bulky ring A moiety.

The presence of four tertiary carbon (CH) signals (all doublets in the coupled spectra), namely at 76.0, 70.8 and 45.0 ppm, support the ketol structure C_1 and C_{12a} (16).



(16)

The three signals to lower field are assigned to C_4, C_5 and C_{12a} since all these carbons are linked to oxygen or nitrogen, while the highest field must arise from C_{4a}. The C_4 and [C_6 methyl] carbons are assigned 63.5 and 13.8 ppm by comparison with corresponding

carbons of TC and anhydro TC respectively.

The spectrum of α -apo OTC may be assigned by analogy. Differences in chemical shifts are mostly small and are most notable at C_3 (shifts to higher field), C_{10} and C_{11} (α shifts to lower field) and C_{4a} (α 34.4 ppm: β 45.0 ppm). Evidence for the exact stereochemical difference between α and β isomers is presented elsewhere (Chapter 2). Detection of apo-OTC impurities in the spectrum of an OTC sample is best made by the observation of the signals to high and low field respectively of the resonance extremes of the parent compound. Chemical shifts in Table 25.

ISO-CHLORTETRACYCLINE BASE

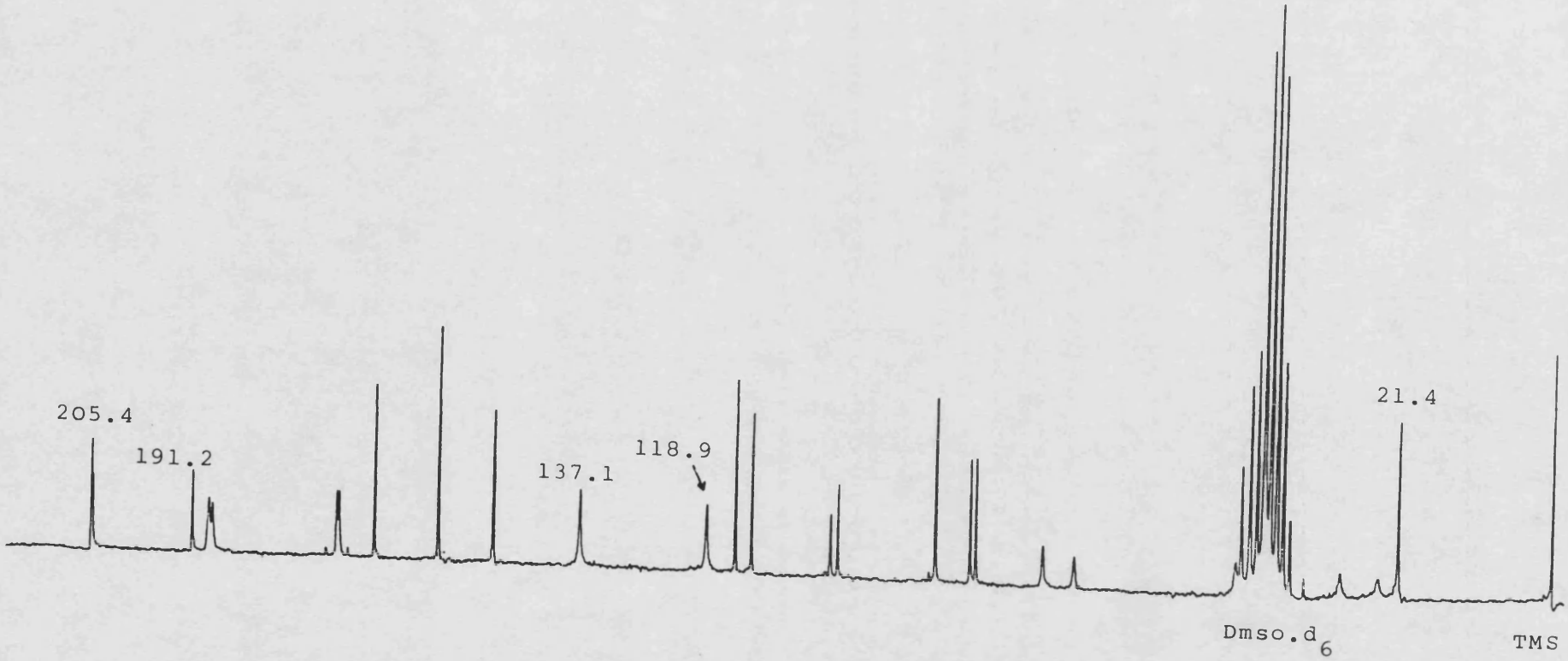
^{13}C NMR (Spectrum 19)

The ^{13}C NMR spectrum of iso CTC was run in Dmso.d_6/TMS at 22.5 MHz. The chemical shifts are listed in Table 27.

17 signals (some duplicated) are observed in the spectral region to low field of the solvent multiplet. Duplications are assumed to be due to the isomeric nature of the product (about C_4).

The lowest field signal pair (205.4, 205.3 ppm) is assigned to C_{12} which is ketonic in the iso CTC formulation (enolic

Spectrum 19. ^{13}C NMR spectrum of iso-CTC base in Dmso.d_6/TMS (22.5 MHz)



in CTC). The 191.2 ppm resonance is assigned to C_1 (ketonic in both iso CTC and CTC) and the 188.9, 188.4 ppm pair to C_3 (probably attached to the ionised OH). The $CONH_2$ carbon resonance of iso CTC (171 ppm) is close to that of CTC (172) The 165.7 and 156.8 ppm resonances are appropriate values for C_{11} (lactone carbonyl) and C_{10} (phenolic) respectively, while the 149.2 ppm resonance may be attributed to C_{6a} (cf. phthalide model, page 222). The 137.0 and 119 ppm resonances(of characteristic broad appearence) must be due to the C_8 and C_9 aromatic carbons of ring **D**, while those at 114.8 and 112.4 ppm probably arise from C_{10a} and C_7 (note C_7 is distinctly low field, cf. CTC 121.4 ppm).

The rest of the low field assignments (see Table 27) follow from comparison with the spectra of CTC and the phthalide model 68 (page 222, for C_6)

The two broad signals (71.1, 66.7 ppm) are attributed to C_4 of the epimers. The high field spectral region displays two CH_2 signals (broad 24.5 and 29.6 ppm) which may be assigned to C_5 and C_{11a} (the iso CTC formulation requires an extra CH_2 feature). Resonances appropriate to C_{4a}, C_{5a}, NMe_2 and C_6-methyl are also present in the spectrum (most clearly revealed by an INEPT experiment).

Table 27. ^{13}C NMR chemical shifts of iso CTC base in Dmso.d_6/TMS. Underlined resonances are obscured by the solvent multiplet. (TMS=0 ppm)

Chemical shifts (ppm)	Carbons assigned
205.4, 205.3	12
191.2	1
188.9, 188.4	3
171.1, 171.0	$CONH_2$
165.7	11
156.8	10
149.2	6a
137.1	8
118.9	9
114.8, 112.4	10a, 7
100.9, 99.9	2
86.1	6
81.3, 80.5	12a
71.1, 66.7	4
<u>44.2</u>	5a
<u>43.2</u>	NMe_2
35.5	4a
29.6, 24.5	11a, 5a
21.4	C_6-CH_3

Section 3

QUANTITATIVE STUDY OF TETRACYCLINES BY ^{13}C NMR

Introduction

Results and discussion

Conclusions

QUANTITATIVE STUDY OF TETRACYCLINES BY ^{13}C NMR

INTRODUCTION

Fourier transform proton decoupled ^{13}C spectroscopy allows the observation of carbon resonances as singlets at the rate of one line per set of equivalent carbon atoms in the investigated molecule. This fact coupled with the wide range of chemical shifts of carbon atoms (0-220 ppm), makes ^{13}C-NMR an attractive tool for analysis of organic compounds or organic mixtures. In quantitative analysis, as opposed to routine qualitative programmes, the necessity for accurate intensity measurements require operating conditions which are quite different from those routinely used. Although the theoretical aspects of quantitative analysis are attractive, the practical steps are quite difficult. These difficulties arise due to the very nature of the ^{13}C nuclei:-

1) The resonance signal is very weak due to the low natural abundance (1.1% of the total carbon), and low magnetic moment of the ^{13}C nuclei.

2) The relaxation times for ^{13}C nuclei are much greater then ^{1}H, consequently there is a significant risk of saturation. Long pulse delays ($5xT_1$) are required which increase the total time taken for the experiment.

3) Nuclear Overhauser Effect (NOE). This results from the broad

band decoupling of protons, practised to simplify the spectrum, which produces an increase in signal intensities which unfortunately is not consistent over the whole ^{13}C chemical shift range. Hence for quantitative work, NOE has to be eliminated e.g. by gated decoupling. This results in very much reduced signal intensity, which in turn makes it very difficult to either measure the signal intensities manually or measure the integration vector.

The accumulation of these problems is responsible for the small number of publications about the quantitative aspects of ^{13}Carbon. One of the solutions around the long spin-lattice relaxation times is to use paramagnetic relaxation agents such as chromium tris(acetylacetonate)(Freeman et al. 1971; Levy and Cargioli 1973). These agents are persumed to shorten long spin-lattice relaxation times and eliminate the NOE. However Levy and Edlund (1975) do not completely agree with this general assumption and recommend that the use of these agents be strongly restricted in quantitative analyses since the NOE's may not be effectively suppressed in the general case. The results obtained in the present study are consistent with the above recommendation.

Kountourellis (1982) worked on the quantitative ^{13}C NMR of gentamycin employing two different types of methods:-

1) Calibration plots of peak height ratios of analyte to internal standard resonance intensities recorded under conditions of full relaxation.

2) Steady state experiment and the use of weighting factors.

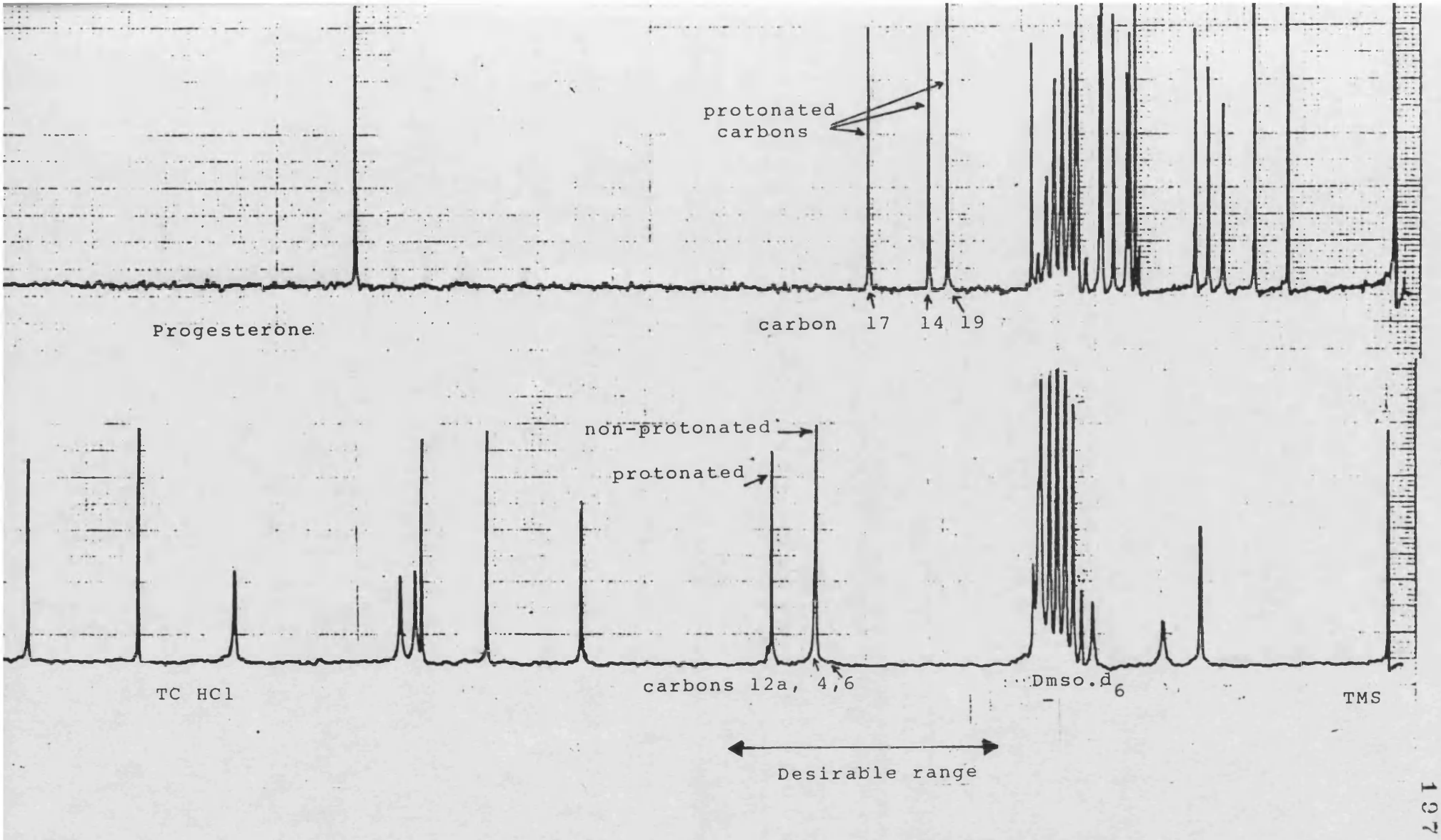
The quantitative work during the present study was carried out using the above method number 1.

RESULTS AND DISCUSSIONS

The initial problem was the choice of a suitable internal standard (IS), which must be of a comparable structure i.e. similar molecular weight and most important similar T_1 values (approximately measured by $W_{1/2}$ where $W_{1/2}$ is the width of the resonance peak at half height). Various anthroquinone derivatives and steriods were tested. Progesterone seemed to give the best results (Spectrum 20). The chemical shift assignments of progesterone are as published by Stothers (1972).

At this stage it is advisable to point out the main reasons for variations in the ^{13}C resonance intensities.

a) R_f pulse may not have enough power to irradiate all the nuclei equally effectively.



Spectrum 20. ^{13}C NMR spectrum of TC HCl and progesterone in Dmso.d_6/TMS. (partial spectrum only)

b) The computer may not have enough data points to completely define all the peaks. The maximum resolution obtained from a pulsed FT spectrum is often determined by the limitations in computer storage rather than magnetic inhomogeneity. If the resolution is such that a given signal is not fully defined by the data points, the resulting signal after transformation may be much reduced. The solution to this problem is to run that part of the spectrum at a much reduced sweep width. This will give, for the same number of data points, a much improved resolution.

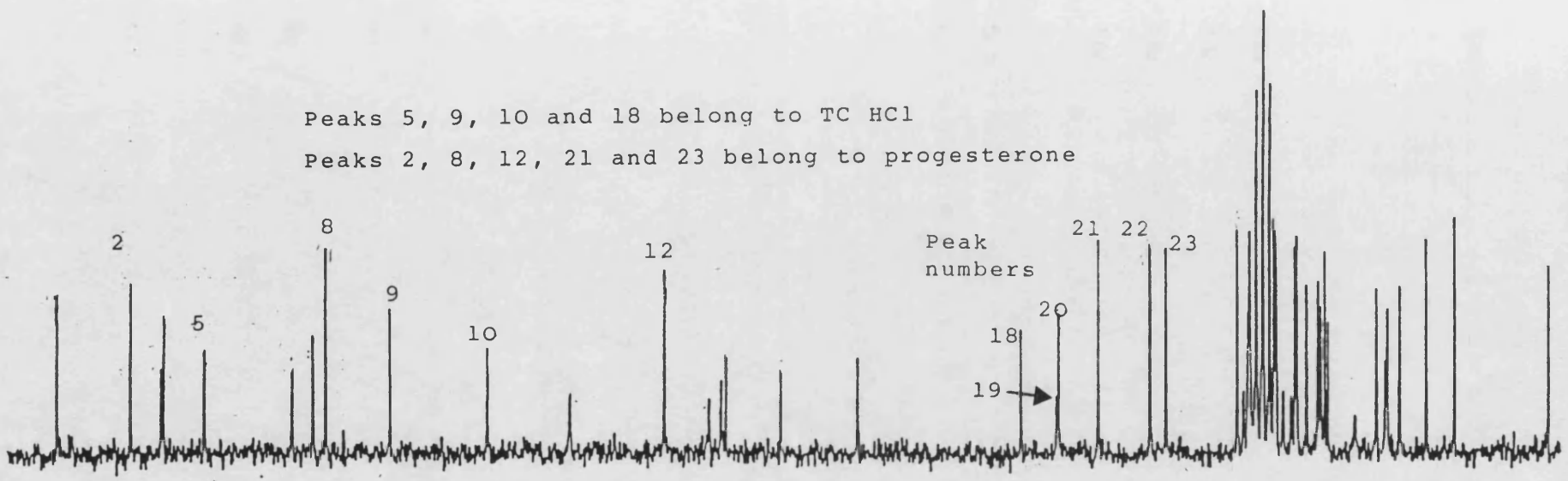
c) Relaxation time variations:- This is by far the most important factor in the large intensity variations observed in the ^{13}C spectrum. One of the solutions to this problem is to insert a long enough pulse delay ($5xT_1$) to allow the carbon atoms to relax back to their equilibrium magnetisation. Since a large pulse delay is employed, a 90^{o} pulse is normally applied to achieve optimum results. One important factor is that, in general the larger the molecule the shorter the relaxation times. Thus these effects become much less severe for larger molecules.

d) Differential NOE effects:- For a carbon relaxing exclusively by dipole- dipole relaxation, NOE has the maximum value of 1.99, the signal intensity will increase by approx. 3 (1+1.99). Gated decoupling is used to remove all NOE.

Since an internal standard (progesterone) is included, it was decided to retain the NOE on the assumption that the enhancement effects would be similar for the analyte and the standard signals.

50.7mg of TC.HCl and 50.0mg of progesterone were dissolved in 0.5 ml of Dmso.d_6. Spectra were run after 2000 scans (Spectrum 21), and data reduction was carried out using exactly the same values (see Table 28). Ratios of analyte/I.S were carried out and standard deviation were calcultated.

As can be seen from the table, the standard deviation values are fairly high, the best values obtained for peak are ratio (18/21) 4.1%.



Spectrum 21. ^{13}C NMR spectrum of a mixture of TC HCl (50.7 mg) and progesterone (50.0 mg) in Dmso.d_6/TMS. Peak area ratios (obtained from the computer print out) and peak height (mm) ratios were measured for the peaks which have been numbered.

Table 28 Peak intensity % ratios and peak height ratios (analyte/I.S.)

Peak area ratios

Pk. No.◆	Run1	Run2	Run3	Run4	Run5	Run6	Standard deviation
18/21	.578	.629	.547	.584	.676	.600	4.1%
18/23	.601	.815	.699	.632	.752	.686	7.1%
10/12	.653	.407	.318	.431	.412	.377	10.5%
9/12	.793	.737	.551	.608	.733	.546	9.71%
5/8	.512	.559	.635	.517	.538	.725	7.6%
5/2	.614	1.04	.612	.818	.697	.760	8.1%

Peak height ratios

◆							
18/21	.396	.649	.530	.586	.650	.591	8.6%
18/23	.512	.847	.678	.654	.776	.650	10.54%
10/12	.667	.370	.304	.386	.437	.409	11.4%
9/12	.813	.720	.518	.621	.775	.561	10.9%
5/8	.511	.537	.634	.505	.539	.769	9.4%
5/2	.608	.945	.604	.803	.700	.702	11.8%

◆ Please refer to Spectrum 21 for Peak numbers.

Data reduction carried out using following computer settings:-

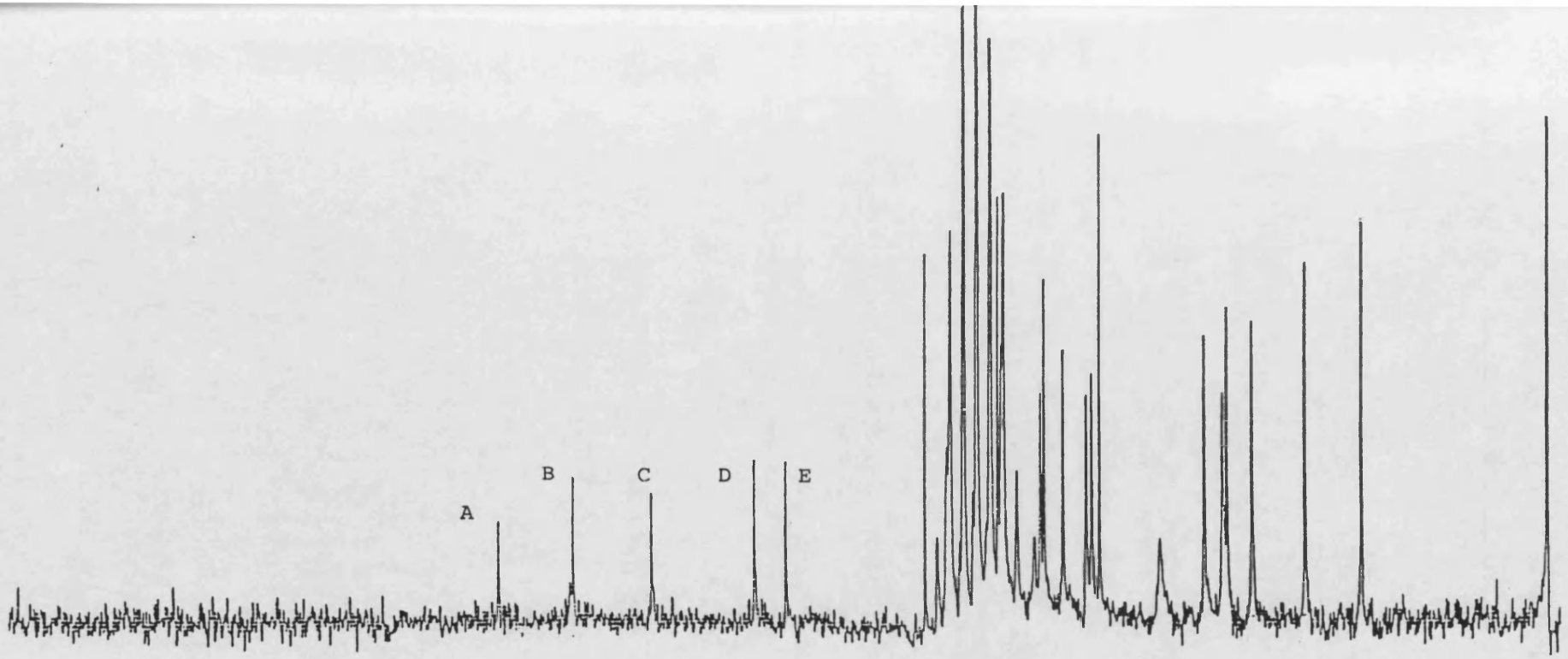
No. of scans 2000, Po 128, BA 1, NL 540, TH 1000, P.d. 1.2 s, pulse width 4 μs.

In the second experiment, a pulse delay of 10s ($5xT_1$) was employed. This should allow enough time for relaxation back to the original level of magnetisation of the spins. A total of 2500 scans were recorded before the spectrum was printed. A total of five different runs were carried out and the standard deviation of the peak height and peak area ratios were calculated. The appearence of the spectra was very poor and often the peaks were not recorded by the computer. The noise level was very high with this experiment. Best standard deviations were calculated using peak height ratios of peaks 9/12 (SD. 0.28%) and peak area ratios of peaks 10/12 (SD. 5.61%).

Since the results were not satisfactory with 10s pulse delay, it was decided to reduce the sweep-width from 5000Hz to 2500Hz. This would allows greater number of data points to define the peaks, although a corresponding greater number of scans have to be accumulated to achieve a reasonable spectrum.

Equal amounts of TC.HCl and progesterone (50mg) were dissolved in Dmso.d_6. A total of 14095 scans were accumulated. Due to its small intensity peak A (Spectrum 22) was often not registered by the computer. Therefore most of the ratios are calculated using peak height measurements. The results obtained are listed in Table 29

Disregarding Run 1, which gave consistently high values, lowest standard deviation of 0.9% was obtained for peak ratio A/G, but this is still higher than the acceptable 0.5% error limit.



Spectrum 22 ^{13}C NMR spectrum of a mixture of TC HCl and progesterone in Dmso.d_6/TMS
Spectral width 2500 Hz.

Table 29. Peak height ratios of TC HCl and Progesterone (50mg of each), dissolved in Dmso.d_6/TMS, sweep width 2500 Hz, 14095 scans, 30° pulse, p.d. 1.2s

	RUN1	RUN2	RUN3	RUN4	RUN5	RUN6	RUN7	S.DEVIATION
A/C ◆	.800	.526	.362	.512	.429	.375	.411	6.3%
A/D	.629	.385	.343	.362	.365	.420	.383	2.4%
A/E	.629	.385	.291	.438	.355	.448	.349	5.4%
B/C	1.11	1.00	.536	.756	.699	.450	.786	17.8%
B/E	.872	.731	.430	.646	.579	.537	.667	9.8%
B/D	.872	.731	.507	.535	.595	.514	.733	9.6%
A/F	.344	.182	.171	.210	.190	.185	.189	1.2%
A/G	.220	.122	.106	.117	.103	.128	.107	0.9%
B/F	.477	.327	.254	.310	.309	.220	.361	4.6%
B/G	.305	.219	.158	.172	.168	.154	.204	2.4%

Up till now all the experimental results were obtained using 5 mm O.D. tubes. It was not possible to increase the concentration of tetracycline or progesterone to more than 50 mg since stronger solutions formed precipitates. It was decided to use 10mm O.D. tubes with 1.5 ml of Dmso.d_6 instead of 0.5 ml previously used. This would allow much more solid to be dissolved resulting in a better S/N ratio. The spectra were all run using ZERO FILL technique.

Four different types of experiments were attempted.

(◆ please refer to Spectrum 22)

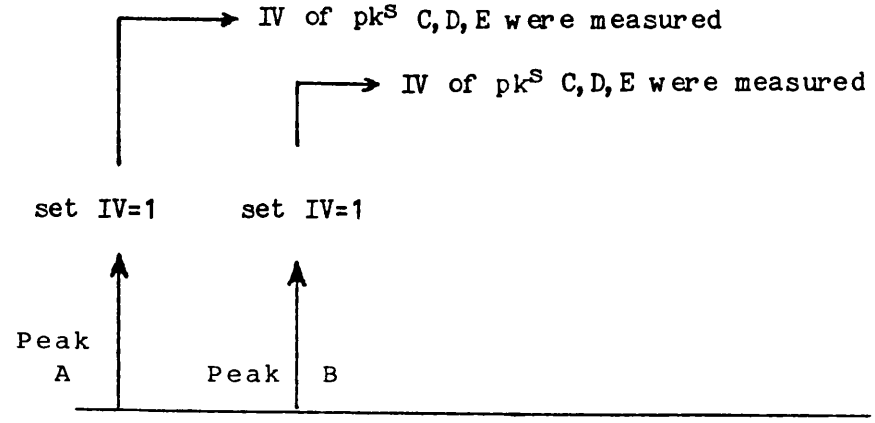
1) 30° pulse, p.d.=1.2s, 1200 scans, complete decoupled spectrum, spectral width 5000Hz.

2) 30° pulse, p.d.=1.2s, 1200 scans, gated decoupled spectrum (NNE), spectral width 5000Hz.

3) 90° pulse, p.d.=10s, 1600 scans, gated decoupled spectrum (NNE), spectral width 5000Hz.

4) 90° pulse, p.d.=10s, 1600 scans, complete decoupled spectrum, spectral width 2500Hz

200mg of both TC HCl and progesterone were dissolved in 1.5m of Dmso.d_6. A few drops of TMS were added. Apart from manually measuring the peak heights, integration vectors (IV) were also used as follows:-



The results obtained in these four experiments are listed in Table 30.

The standard deviations obtained with experiment 1 were fairly high

Table 30

Integration vectors (IV) for TC and progesterone peaks. Peaks A and B belong to TC HCl and peaks C,D and E are due to peogesterone. (for reference see Spectrum 22). IV of peak A is set to 1.0 and the IV values for the peaks C,D and E are recorded. Similarly with peak B.

1)	A/C	IV pk C	A/D	IV pk D	A/E	IV pk E	B/C	IV pk C	B/D	IV pk D	B/E	IV pk E	
30° pulse, p.d 1.2s 1200 scans	.6364	8.2	.5833	5.8	.5833	8.9	.9091	.46	.8333	.61	.8333	.94	....Run 1
	.6591	2.4	.6744	3.3	.6824	3.4	.7955	1.0	.8140	1.6	.8235	1.4	....Run 2
	.5429	1.0	.5588	1.0	.5938	1.3	.8286	.79	.8529	.85	.9063	.99	....Run 3
COM	.5714	1.39	.5455	1.34	.6154	1.76	.6429	1.04	.6136	.92	.6923	1.23	....Run 4
5000 Hz	.6000	2.25	.5647	1.65	.5783	2.28	.8750	1.18	.8235	.89	.8434	1.21	....Run 5
Standard deviation	4.2%	$\bar{x}$ 3.1	4.6%	$\bar{x}$ 2.6	3.8%	$\bar{x}$ 3.5	9.2%	$\bar{x}$ .9	8.8%	$\bar{x}$ .98	6.99%	$\bar{x}$ 1.2	
2)													
30° pulse, p.d. 1.2s, 1200 scans	1.063	.97	1.214	.98	1.133	.77	1.125	.76	1.286	.56	1.200	.60	...Run 1
	.8824	1.4	1.154	1.0	1.154	1.0	1.088	1.2	1.423	.74	1.423	.84	...Run 2
	1.125	1.4	1.125	.89	1.500	1.1	1.125	.57	1.125	.65	1.500	.68	...Run 3
NNE	.6154	1.1	.6154	.62	.6316	1.1	.9487	.68	.9487	.50	.9737	.81	...Run 4
5000 Hz	.8654	1.0	.7500	.82	.9000	.83	1.269	.84	1.100	1.1	1.320	---	...Run 5
Standard deviation	17.8%	$\bar{x}$ 1.2	24.2%	$\bar{x}$ .86	28.9%	$\bar{x}$ .96	10.2%	$\bar{x}$ .81	16.3%	$\bar{x}$ .71	18.5%	$\bar{x}$ .73	
3)													
90° pulse, p.d. 10s, 1600 scans,	.5882	.98	.5882	1.1	.5882	1.0	.8529	1.4	.8529	1.1	.8529	1.1	...Run 1
	.5714	---	.5925	---	.5517	---	.9286	---	.9629	---	.8966	---	...Run 2
	.4762	.75	.4546	.72	.4546	.63	.9286	1.0	.8864	.83	.8864	.88	...Run 3
NNE	.5000	---	.5000	---	.5000	---	.9091	---	.9091	---	.9091	---	...Run 4
5000 Hz	.4828	1.0	.4667	1.2	.5000	.79	1.103	.90	1.067	.51	1.143	.56	...Run 5
Standard deviation	4.7%	$\bar{x}$ .91	5.9%	$\bar{x}$ 1.0	4.6%	$\bar{x}$ .81	8.42%	1.1	7.5%	$\bar{x}$ .81	10.43%	$\bar{x}$ .85	
4)													
90° pulse, 10s p.d., 1600 scans,	.4688	---	.2459	---	.1667	---	.5246	---	.4849	---	.3556	---	...Run 1
	.3171	---	.2766	---	.2600	---	.5366	---	.4681	---	.4400	---	...Run 2
	.3333	---	.2609	---	.2400	---	.6667	---	.5217	---	.4800	---	...Run 3
COM	.2958	---	.2308	---	.2593	---	.4648	---	.3626	---	.4074	---	...Run 4
2500 Hz	.3600	---	.3000	---	.3000	---	.5200	---	.4333	---	.4333	---	...Run 5
Standard deviation	6.7%		2.7%		4.9%		7.5%		6.0%		4.6%		

(range 3.8% to 9.2%). This may be due to :-

a) Differential NOE effects.

b) Longer pulse delay required.

c) Bigger pulse required.

d) Progesterone is not a suitable internal standard.

To eliminate (a), programme 2 was run on the same sample but under gated decoupled conditions. The standard deviation values were even worse than before (range 10.2% to 28.9%). The peak heights were very low along with S/N ratio, increasing the percentage error in peak height measurements. Programme 3 and 4 were run to employ conditions (b) and (c) stated above. Since largest T_1 values of TC HCl for peak A and B (Chapter 2) is 2s, a pulse delay of 10s should be sufficent to allow complete relaxation. The standard deviation values obtained for programme 3 were still too high (range 4.7% to 10.43%), but IV values were the best ones achieved so far (range 0.81 to 1.1 expected value=1.0).

The fourth programme was run with a sweep width of 2500Hz. This allows twice the data points for half the spectrum. No improvement in the standard deviation was noticed (range 2.7 to 7.5%), and since the peaks were twice as broad it was impossible to integrate them. One final programme was run with same experimental conditions as programme 4 except sweep width was 5000Hz. The standard deviation results were in the range 7.0 to 11.6%). No consistent IV values

were obtained.

CONCLUSIONS

It is clear from all the results obtained that ^{13}C is not the method of choice for quantitative measurements. There are so many different variables involved, that it is very difficult to obtain consistent readings.

From the experimental data , the following points need to be considered very carefully before starting quantitative measurements by ^{13}C NMR.

1) Choice of a suitable internal standard:-

The compound should be of a similar structure and molecular weight.

Thepeaks chosen should have similar splitting pattern to that of the analyte i.e. both of the carbons should either be protonated or non-protonated.

2) Concentration in solution:-

It is best to dissolve as much solid as posssible depending on solubility. 10mm O.D. tubes are recommended.

3) Pulse delay:- The pulse delay should be large enough to allow

complete relaxation e.g. 5 x T_1.

4) Pulse width:- If a large enough pulse delay is used it is best to use 90° pulse to obtain optimum magnetisation.

5) Complete or Gated decoupled spectrum:-
It is difficult to decide between the two. Complete decoupled (COM) spectrum allows differential NOE, but with the gated decoupled (NNE) spectrum, the number of scans accumulated have to be increased considerably.

6) Zero filling:- This technique allows more data points for the resolution spectrum. If possible this should be employed.

7) Instead of measuring peak height intensities manually, measuring the integration vector by computer is recommended, since the error in base line setting would be constant.

Finally, some work was carried out with the relaxation agent Cr acac. Using 0.1M Cr acac, the T_1 values were very much higher than those of the pure sample, but using 0.05M Cr acac the T_1 values dropped considerably. Since no consistency was achieved using Cr acac, further work was not attempted using relaxation agents.

CHAPTER 5

STANDARDS AND REFERENCE COMPOUNDS

Phenol

p-Chlorophenol

p-Aminophenol

Aniline

N,N,-dimethylaniline

1,8-dihydroxynaphthalene

Benzene sulphonic acid (as anion)

p-Toluene sulphonic acid (Na salt)

Chromotropic acid

Dithranol

Phthalide

2-Carboxamidodimedone

STANDARDS AND REFERENCE COMPOUNDS

As reference compounds play a major role in the assigning of the ^{13}C NMR chemical shifts of tetracyclines, it was considered important to group all the reference compounds in one section, so that the chemical shift assignment evidence could be referred, as appropriate, to any particular compound.

Literature data on many of the reference compounds are available in non-polar solvents e.g. cyclohexane etc. Since the tetracyclines require polar solvents, spectra of many of the simple compounds used as standards have been examined in Dmso.d_6 to determine the magnitude of the solvent effect. Benzene is taken as the starting point for shifts of the phenolic ring of tetracyclines (ring D); the ^{13}C chemical shift of benzene is 128.4 ppm from TMS in Dmso.d_6. Unless indicated otherwise, all the chemical shifts are in ppm with TMS = 0 ppm. In reporting shielding influences of a substituent a +ve value indicates deshielding (move to a larger chemical shift) and a -ve value indicates shielding (move to a smaller chemical shift). The chemical shifts data is summarised in Table 31 .

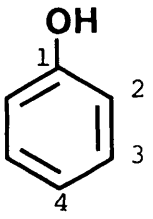
PHENOL

The hydroxy group attached to benzene has the following influences

Table 31. ^{13}C NMR chemical shifts of some mono and disubstituted benzene derivatives in Dmso.d_6, in ppm fro TMS (benzene 128.4 ppm)

Substituent	C_1	C_2	C_3	C_4	C_5	C_6	Others
OH	156.8	115.9	129.9	120.3	129.9	115.9	
1-OH,4-Cl	156.4	117.1	129.2	123.1	129.2	117.1	
NH_2	148.2	114.7	129.1	116.8	129.1	114.7	
NMe_2	150.7	112.6	128.9	116.4	128.9	112.6	Me_2 40.1
1-OH,4-NH_2	148.6	115.8	115.8	140.4	115.8	115.8	
after NaOH	161.2	119.5	119.0	134.0	119.0	119.5	
SO_3 Ca salt	147.1	125.5	127.7	128.9	127.7	125.5	
1-SO_3,4-Me	144.7	126.0	128.5	138.7	128.5	126.0	Me 21.0
(Na salt)							

deshields	C_1 strongly	(+27.2, 156.8 ppm)
	C_3 weakly	(+1.8, 129.9 ppm)
shields	C_2 strongly	(-12.6, 115.1 ppm)
	C_4 strongly	(-7.9, 129.9 ppm)



The off-resonance spectrum of phenol in Dmso.d_6 showed, as expected, one singlet and one doublet of low intensity and two doublets of relatively greater intensity (due to the equivalent C_2,C_6 and C_3,C_5). The singlet at 156.8 ppm is assigned to C_1 and the doublet with lower intensity at 120.3 ppm is assigned to C_4. The OH group shields C_2 the most thus pushing the signal upfield, and deshields C_3 thus displacing the signal downfield. Hence the doublet at 115.9 ppm is assigned to C_2 and that at 129.9 ppm to C_3. Values quoted by Stothers (1972) were obtained from cyclohexane solutions and are very similar to the ones obtained in Dmso.d_6. Therefore the solvent effect is seen to be negligible.

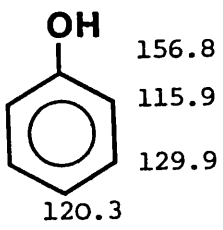
	Dmso.d_6	Cyclohexane
C_1	156.8 ppm	155.6 ppm
C_2	115.9	116.1
C_3	129.9	130.5
C_4	120.3	120.8

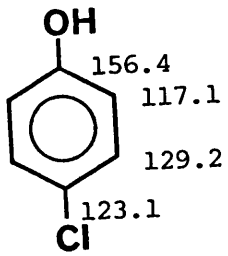
p-CHLOROPHENOL

The chlorine atom influences aromatic carbons (Stothers, 1972) as follows:

deshields C_1 (+6.4 ppm), C_2 (+0.2 ppm) and C_3 (+1.0ppm), but shields C_4 (-2.0 ppm). Therefore the effect of inserting a chlorine atom para to the hydroxy group of phenol is predicted to move C_1 and C_2 of phenol upfield by a few ppm and C_2, C_4 downfield by a few ppm and to have a negligible effect on C_3.

The results obtained were as expected, except for the small magnitude of the shift seen at C_1. The chemical shifts are presented in Table 31.



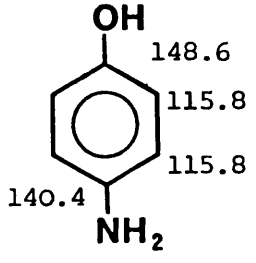


p-AMINO PHENOL

The amino group deshields C_1, C_3 and shields C_2, C_4 (61, page 148) i.e. it behaves very similar both in direction and order of effect to the hydroxy group. Insertion of an amino group, para to OH of phenol, is thus predicted to move the C_1, C_3 resonances significantly upfield (9-13 ppm) and effect a large downfield shift of C_4 and a small downfield shift of C_2. (Ring numbers apply to phenol).

The spectrum showed an intense resonance at 115.8 ppm in Dmso.d_6 (doublet in the coupled spectrum) which is assigned to C_2, C_6 and C_3, C_5 carbons (the signals overlap, but separate when the OH is ionised, see below). A problem arises over the assignment of the two singlets at 148.6 and 140.4 ppm. Since the shielding effects of OH and NH_2, upon the para carbon, are very close in magnitude it is probable (from C_1 shifts of phenol and aniline) that the lower field resonance is due to the phenolic carbon. Spectral shifts after ionization of the phenolic group confirm these assignments.

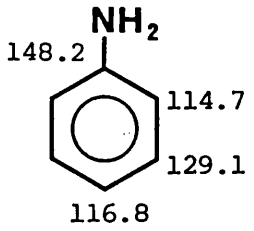
	Dmso.d_6	Dmso.d_6 + NaOH/H_2O	
C_1	148.8 ppm	161.2 ppm	+13.4
C_4	140.5	134.0	-6.6



The magnitude of shifts of C_1 and C_4 resonances obtained after ionization are close to those of phenol itself (ionization substantially augments the shielding effects at these two positions (Stothers, 1972)). The alternative assignments of C_1 (140.5 ppm) and C_4 (148.8 ppm) gives $\Delta\delta$ values that are too large.

ANILINE

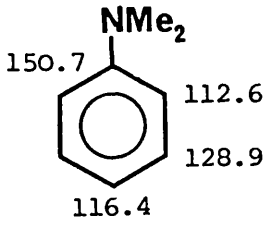
As stated above, in the case of p-amino phenol, the amino group deshields the C_1 (+19.2), C_3 (+1.3) carbons and shields C_2 (-12.4), C_4 (-9.5). The spectrum showed two high intensity doublets, one low intensity doublet and a single. The following values are hence assigned. The chemical shifts are listed in Table 31



Aniline

N,N-DIMETHYL ANILINE (42)

This model is useful for the assignments of minocycline. The spectrum of the model compound, in Dmso.d_6, was readily assigned upon comparison with that of aniline in the same solvent. It is seen that extra methyl substituents have small effects at C_1 (2.5 ppm-deshielding), C_2 (2.1 ppm -shielding) and negligible effects at C_3 and C_4.The chemical shifts are listed in Table 31.



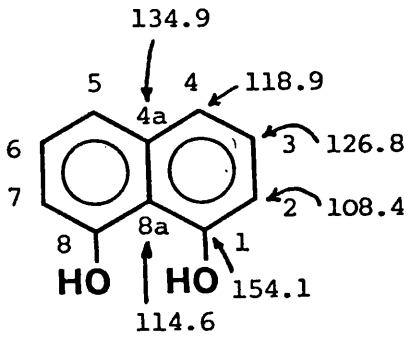
(42) N,N-dimethyl amine

1,8-DIHYDROXYNAPHTHALENE (56)

The spectral assignments of phenol have to be considered to assist the analysis of the model (56). In the case of 1,8-dihydroxy naphthalene, the major spectral difference would be at the C_{8a} and C_{4a} carbons since they suffer double shielding effects).

In the spectrum run in Dmso.d_6, three doublets appear at 126.8, 118.9

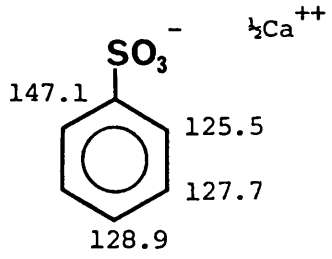
and 108.4 ppm which are assigned to C_3,C_6; C_4,C_5 and C_2,C_7 respectively on the basis of shifts for phenol. These values differ somewhat from those of corresponding phenol carbons, especially the C_2,C_7 shift. This may be a result of ortho deshielding due to the OH group and para shielding via four bonds due to the second OH group. hence the signal shifts upfield. A similar but weaker effect is seen at C_4,C_5. C_{4a} is deshielded by both OH groups whereas C_{8a} is shielded. Therefore the singlet at 134.9 ppm is assigned to C_{4a} and that at 114.6 ppm to C_{8a}. The lower field singlet (154.1 ppm) is clearly associated with the carbon directly linked to OH. The chemical shift data is listed below.



(56) 1,8-dihydroxy naphthalene

BENZENE SULPHONIC ACID (AS ANION) (70)

It was required to determine the influence of a sulphonic acid group on the aromatic carbon resonances to aid assignments of chromotropic acid (see later).

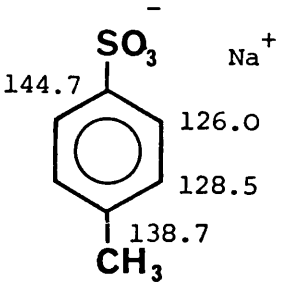


(70)

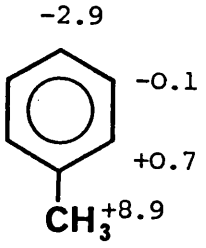
The spectrum of the calcium salt, in Dmso.d_6, displayed one singlet, one low intensity doublet and two high intensity doublets close together. The singlet must be due to C_1 and the low intensity doublet is assigned to C_4. The two high intensity doublets are too close together for precise assignment and may be interchanged. Therefore the sulphonic acid has mainly deshielding effect (+ 18.4 ppm) at C_1 and its influence on other carbons is negligible. The chemical shifts are listed in Table 31.

p-TOLUENE SULPHONIC ACID (Na Salt) (71)

Assignments of the spectrum of the sodium salt of this acid and shifts seen for the lower homologue (70) are consistent with the shielding influence of the methyl group on the benzene ring (72)



(71)



influences of CH_3 group
(+ deshielded, - shielded)
(benzene 128.4 ppm)
(72)

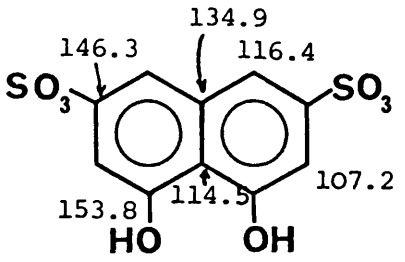
(Abraham and Loftus, 1980)

Hence the effect of the sulphonic acid may be summarised as follows:

deshields:	C_1	+18.4 ppm
	C_4	+0.2
shields:	C_2	-3.2 ppm
	C_3	-1.0

CHROMOTROPIC ACID (73)

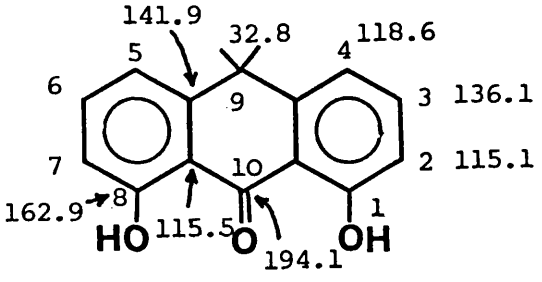
The sulphonic acid would predominantly affect the C_3 and C_6 chemical shifts. The rest of the assignmnets should be similar to 1,8-dihydroxy naphthalene, as described earlier. Hence, by comparison the following assignments are confirmed.



(73) Chromotropic acid

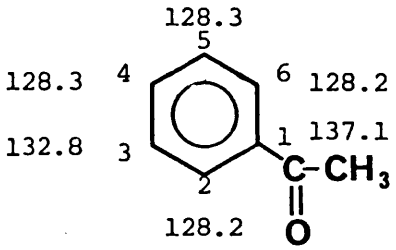
DITHRANOL (55)

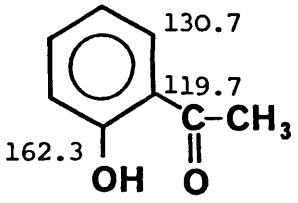
Dithranol is described in the Merck Index (8th edition) as 1,8,9-trihydroxy anthracene, but its ^{13}C spectrum, in Dmso.d_6, clearly shows it to have 1,8-dihydroxy-9-keto structure (55). The low field resonance at 194.1 ppm is typcal of carbonyl carbon and the high field signal at 32.8 ppm is typical of a methylene carbon.



(55) dithranol

Of the four singlets, that at 194.1 ppm is assigned to C_{10} (carbonyl) and that at 162.9 ppm to the phenolic carbons (C_1, C_8). Differentiation between the C_{8a}, C_{9a} and C_{4a}, C_{10a} may be made by reference to the chemical shifts of acetophenone (53) and 2-hydroxy acetophenone (52).





(53) (52)

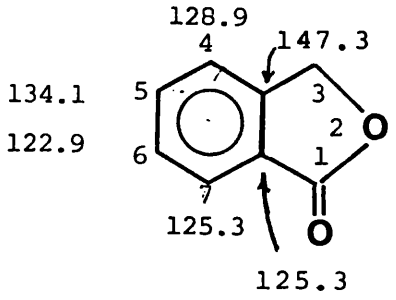
(^{13}C data bank, 1976)

A methyl substituent at C_6 of (52) would shift the C_6 resonance downfield, hence the assignments of dithranol may be confirmed as as shown above.

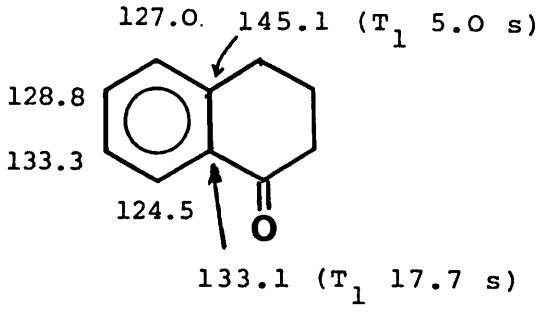
PHTHALIDE (68)

Phthalide is a suitable model for the apo derivatives of the oxytetracycline antibiotics. The spectrum, run in Dmso.d_6, displayed the expected eight signals. On the basis of the α-tetralone

assignments ([74] , confirmed by T_1 experiment), the singlet at 147.3 ppm is assigned to C_{3a} and that at 125.3 ppm to C_{7a}. Methine carbon shifts are allocated by similar comparison.



(68)

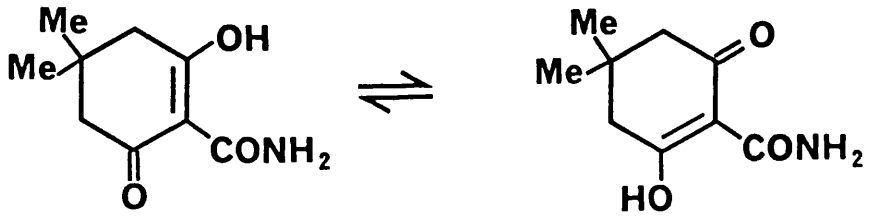


(74)

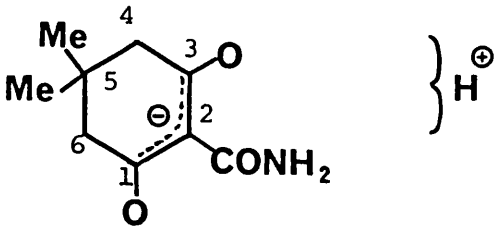
α-tetralone

2-CARBOXAMIDODIMEDONE

The 2-carboxamidodimedone was prepared in the laboratory following the method of Muxfeldt et al. (1966) as described on page 43. The compound can exsist in two tautomeric forms as shown below. The ^{13}C NMR spectrum was run in Dmso.d_6/TMS and the assignments are discussed with reference to the structure (24).



(24a) (24b)

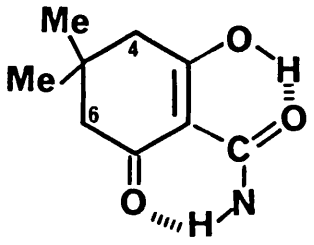


2-carboxamidodimedone (24)

Following chemical shifts are assigned.

C_1, C_3	195.9 ppm
$CONH_2$	173.3 "
C_2	100.2 "
C_4, C_6	50.6, 45.7 ppm
C_5	30.4 "
Methyl	27.7 "

Observation of the two signals for C_4 and C_6 is evidence that the rate of tautomeric interconversion of (24a) and (24 b) must be slower than the NMR experiment. Each tautomer may be stabalised by a pair of intramolecular hydrogen bonds as shown below (24c). In (24 c) the C_4 and C_6 are magnetically non-equivalent.



(24c)

CHAPTER 6

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Section 1

Solvent optimisation with four solvents to analyse tetracyclines

Introduction

Theory for solvent optimisation in HPLC

Experimental

Results and discussion

Evaluation of the seven chromatograms

Conclusions

pH profile of separation 5

Summary

SOLVENT OPTIMISATION WITH FOUR SOLVENTS TO ANALYSE TETRACYCLINES

INTRODUCTION

During the past few years, numerous papers have been published on the chromatographic separation of the tetracycline group of drugs (Butterfield et al. 1975; De Leenheer et al. 1977; Mack and Ashworth, 1978), and of their common impurities. Their assay in biological fluids such as urine and blood, following therapy (Sharma et al. 1977) has also been described. The packing material used in all of these studies has been silica-based, employing aqueous-organic eluents in the pH range 1-2.5. There are two main disadvantages using this pH range.

1) Silica-based materials are unstable below pH 2.0 and above pH 8.0, because of hydrolysis of the hydrocarbon surface and dissolution of the silica respectively.

2) As demonstrated in the pH stability study (see page 272), tetracyclines degrade rapidly to anhydro derivatives at low pH. At the other end of the pH scale, degradation results in the formation of the apo-derivatives.

Hence, there is clearly a need for an analytical method using mild pH conditions. Knox and Jurand (1979) published a method using SAS-Hypersil packing material in the pH range 3-5. EDTA was necessary for good separation. Two organic modifiers were tested, acetonitrile and dimethylformamide of which the latter was found to be superior.

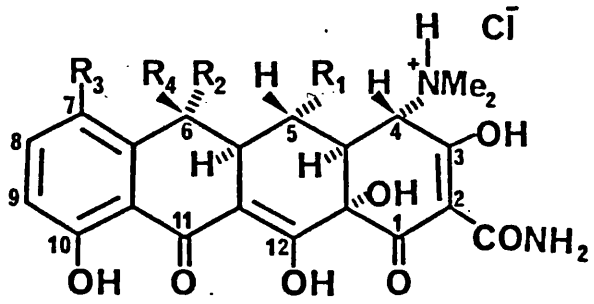
Studies employing mobile phases of higher pH have been described using reversed phase (Tsuji and Robertson, 1976) and ion-exchange (Lotsher et al. 75), although none attempted to separate all the compunds listed in Table 32. The recent introduction of the polystyrene divinybenzene material (PRP-1) provided an opportunity to investigate a significantly different column material with a view to providing a more selective chromatographic system for as many tetracyclines as possible, without employing gradient elution. This system would then be used to compliment the ^{1}H and ^{13}C NMR spectroscopy being used for the analytical and structural investigation of the tetracycline antibiotics and their impurities.

In order to reduce the number of compounds studied in the optimisation of the chromatographic conditions, representative compounds were selected. Tetracycline (TC) and 6-demethylchlortetracycline (demethyl CTC) represent compounds without an OH group at C_5, but having different functionalities at C_6 and C_7. Oxytetracycline (OTC) and methacycline represent compounds possessing an OH group at C_5, again having different groups at C_6 and C_7. Minocycline was also chosen because of its additional NMe_2 at C_7. The main aim of the present work was to develop a simple analytical method using mild pH conditions and to investigate the retention mechanisms involved.

The structures of the main tetracyclines and their common impurities are presented in Table 32. It may be noted that the tetracycline

skeleton is rigid. Most of the members of the family possess a great number of functional groups with varying pKa values. The degradation products are often isomers with only minor structural differences from the parent compound.

Table 32: Structures of Tetracycline and other related drugs



	R_1	R_2	R_3	R_4
Tetracycline (TC)	H	CH_3	H	OH
Oxytetracycline (OTC)	OH	CH_3	H	OH
Chlortetracycline (CTC)	H	CH_3	Cl	OH
Minocycline	H	H	H	H
Methacycline	OH	CH_2	H	-
Doxycycline	OH	CH_3	H	H
6-demethyl CTC	H	H	Cl	OH

At this stage, it would be appropriate to give a brief account of the underlying theory for using four solvents in HPLC.

In order to optimise the mobile phase composition for the PRP-1 material, it was decided to evaluate the separations obtainable using combinations of up to four different solvents, as described by Glajch et al. (1980). This approach has not been described in the literature for this material and it was felt that useful changes in selectivity could be obtained.

THEORY FOR SOLVENT OPTIMISATION IN HPLC

The quality of an HPLC analysis is assessed by measurement of the resolution between adjacent peaks:-

$$R_s = \frac{1}{4} * \sqrt{N} * \frac{K_2}{(1 + K_2)} * \frac{(\alpha - 1)}{\alpha} \qquad \alpha = \frac{K_2}{K_1}$$

$$R_s = \frac{t_{r2} - t_{r1}}{\frac{1}{2}(w_2 + w_1)}$$

The three factors contributing to resolution are column efficiency (N), solute retention (K) and selectivity (α); each term may be adjusted independantly to achieve adequate R_s values. This is generally required to be not less than 1.0, equivalent to 98% separation of two components. Efficiency (N) is mainly a function

of the column and the way it is packed. The column used in these studies was purchased from Hamilton U.k. The solute retention (K) is dependent on solvent strength and solvent composition. As a rule, stronger solvents reduce K values, whereas weaker solvents increase K values. Initially, a choice of mobile phase was made to yield a range of kappa values between 1-10.

The selectivity factor (α) is a measure of the chromatographic system to recognise chemical differences between the two components. It is similar to the measurement of resolution but does not take peak width into account, only retention times. Values must be greater than 1.0.

The properties of HPLC solvents resposible for the solute-solvent interactions that produce solute elution from the column may be described (Glajch et al. 1980) as the relative ability of each solvent to inter-act as a proton-donor, proton-acceptor or dipole. These properties are described as X_d, X_e and X_n respectively, because dioxane, ethanol and nitromethane were chosen as test solutes to measure these properties. The X values describe the relative proportions of these three properties for any solvent. These properties largely explain the different elution behaviour of different solvents. In a solvent optimisation study, it is best to examine a few solvents having widely different X_d, X_e and X_n ratios, to discover selectivity differences. Retention is adjusted by taking into account the total polarity of the solvent as described by the term P' (Snyder, 1974). In 1978, Snyder plotted the selectivity

differences as measured by X_d, X_e and X_n values of 81 solvents and showed that they were distributed into groups of solvents, generally of similar chemical type. The opportunities for discovering selectivity differences obtainable by using different solvents is greatly enhanced by examining one typical solvent from three widely differing groups. For reversed-phase systems, methanol, acetonitrile and tetrahydrofuran were recommended (see Fig. 13).

In this study, propan 2 ol was substituted for methanol because it produced narrower peaks. Each solvent was then mixed with the volume of water that would produce a chromatogram in which the components of the drug mixture would have kappa values in the range 1 to 10. These compositions may then be further blended as described in Fig. 14. to yield a total of seven different compositions, which may then be assessed to choose the optimum system. Recently this approach has been computerised, but this was not available to us.

Fig. 13 Grouping of solvents by selectivity.

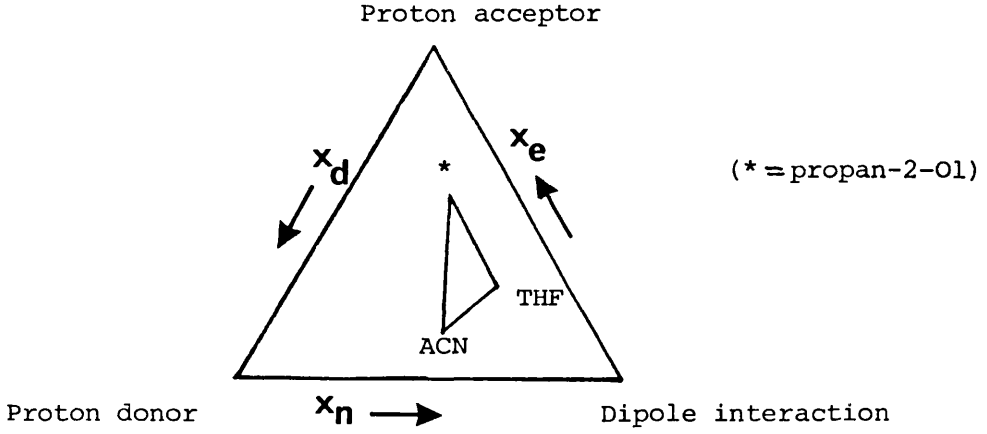
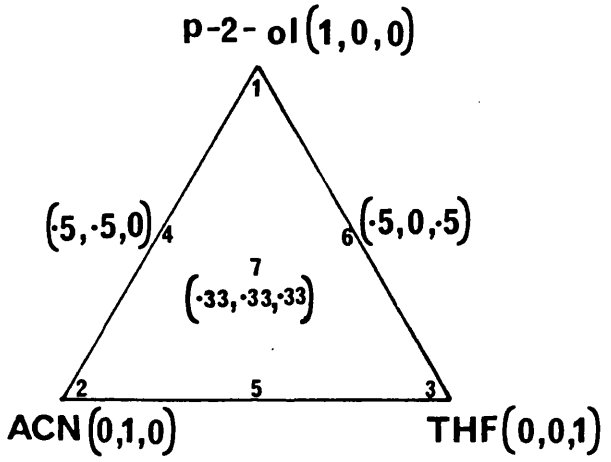


Fig: 14 Optimization triangle defining seven experiments and mobile phase blend ratios



EXPERIMENTAL

The three organic modifiers used were acetonitrile (ACN), tetrahydrofuran (THF) and propan-2-ol, all HPLC grade supplied by Fisons U.K. All the salts used in preparing the buffer system were Analar grade and used without further purification.

Buffer Composition:-

Solution A :- $Na_2HPO_4.\ 2H_2O$ = 8.903 g (0.05M)

Citric acid. H_2O = 7.00 g (0.033M)

H_3BO_3 = 3.54 g (0.0507M)

NaOH 1M solution = 243 ml

The mixture was made up to 1L with double distilled deionised water.

Solution B :- 0.1 M HCl solution

The buffer solution was prepared by mixing 40 ml of solution A and 110ml of solution B and adding water to make up to 200ml. The pH of this solution mixture, without the organic modifier, was 5.00. All the buffer solutions were prepared in exactly the same way. A single piston pump was employed with a flow-rate of 1.1 ml/min. The column (25cm, PRP-1), 10µl loop and the mobile phase reservoir were all kept in a water bath at 42°C maintained by a thermostirrer (Gallenkamp). A UV-VIS variable wavelength detector (Du Pont) operating at 272nm was

used at AUFS 0.08. Chart speed was maintained at 10 mm/min. A BBC chart recorder was used.

RESULTS AND DISCUSSION

The first stage in the experiment was the development of the three binary solvent compositions i.e. the percentage composition of one organic modifier plus buffer to achieve acceptable kappa values between 1-10. Since gradient elution facilities were not available, the percentage composition of the organic modifier was by trial and error. In the first case, 22% propan-2-ol plus buffer was found to be optimum, although resolution was not good the kappa values were in in the desired range of 1-10.

To calculate the equivalent concentrations of the two remaining organic solvents data published by C. Riley (1980) was used. Following compositions were chosen:-

THF = 14.2 % and ACN = 20 %

Having established the proper binary solvent compositions for the apexes of the optimization triangle (Fig. 14), the use of standard ratios for positions 4,5,6 and 7 provided the remaining four mobile phase compositions. For the tetracyclines, mobile phases for the seven definite experiments are shown in Table 33.

Table: 33 Various blend ratios and mobile phase compositions

Separation	Blend ratio ACN:	THF:	Propan-2-ol	Mobile phase composition ACN:	THF:	Propan-2-ol :	Buffer
1	1.0	0.0	0.0	20	0.0	0.0	80.0
2	0.0	1.0	0.0	0	14.3	0.0	85.8
3	0.0	0.0	1.0	0	0.0	22.0	78.0
4	0.5	0.5	0.0	0	7.1	0.0	82.9
5	0.0	0.5	0.5	0	7.1	11.0	81.9
6	0.5	0.0	0.5	10	0.0	11.0	78.0
7	0.33	0.33	0.33	6.6	4.7	7.3	81.4

EVALUATION OF THE SEVEN CHROMATOGRAMS

With the first three solvent systems (1,2 and 3), the individual drugs were injected on to the column, followed by an injection of a mixture containing all the individually injected drugs. The following drugs were included in the mixture:- 1) OTC; 2) TC; 3) demethyl CTC; 4) methacycline; 5) minocycline

The chromatographic results of the first three solvent systems are listed in Tables 34, 35, and 36 ; the chromatogram of separation 1 is shown in Fig. 15.

Table: 34 Chromatographic results with solvent system 1

		t_r (mm)	K	$W_{1/2}$ (mm)	Peak symmetry
1	OTC	21.0	1.10	2.0	1.0
2	TC	25.2	1.52	2.0	1.0
3	demethyl CTC	33.3	2.33	3.0	1.0
4	methacycline	37.5	2.75	3.0	1.16
5	minocycline	73.0	6.30	12.0	0.91

Table: 35 Chromatographic results of solvent system 2

2	TC	24.0	1.4	--	--
1	OTC	28.0	1.8	--	--
3	demethyl CTC	37.0	2.7	--	--
4	methacycline	52.5	4.25	5.5	0.59
5	minocycline	73.5	6.35	12.5	0.6

Table: 36 Chromatographic results with solvent system 3

1	OTC	17.0	0.7	--	--
2	TC	17.0	0.7	--	--
3	demethyl CTC	24.0	1.4	--	--
4	methacycline	31.0	2.1	--	--
5	minocycline	59.0	4.9	--	--

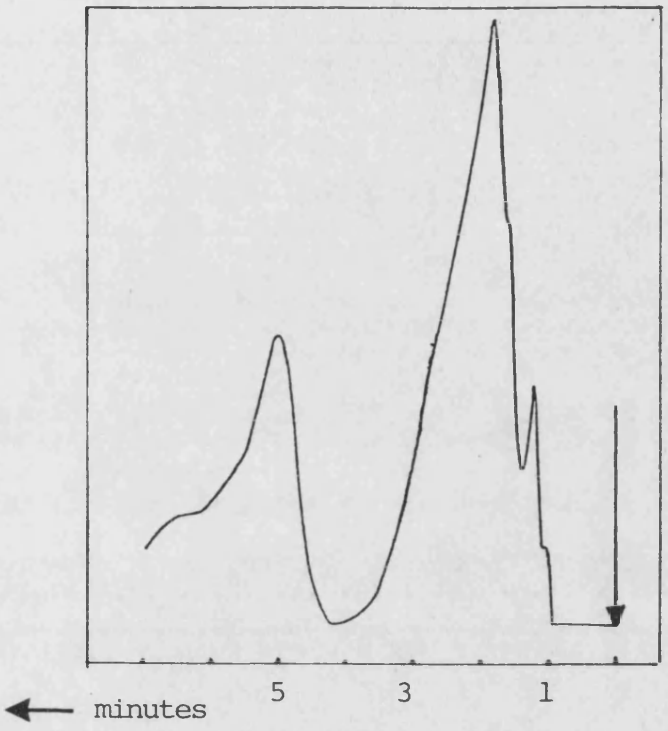


Fig. 15. Isocratic separation of tetracyclines with solvent system 1.

Operating conditions

Column PRP-1 25cm

Mobile phase:-

Propan-2-ol	0%
THF	0%
ACN	20%
Buffer	80%
Temperature	42°C

The elution order plus changes in that order may also be seen from Fig. 16 A comparison of separations of solvent systems 1,2 and 3 (Tables 34, 35 and 36 and Fig. 15) reveal that the separation with only one of the organic modifier present is not adequate due to very poor resolution. Furthermore, a reversal of order between OTC and TC can be observed between solvent systems 1,2 (Fig. 16); while in separation 3, both OTC and TC elute together.

Solvent sytems 4, 5 and 6 are combination mobile phase systems containing two organic modifiers in various proportions (Table 33). The chromatograms plus other data are shown in Fig. 17, 18 and 19 for solvent systems 4,5 and 6 respectively.

Separation 4 (Fig. 17) produced no elution reversal compared to separation 3; but apart from components 1 and 2 (OTC, TC), the other three components are fully resolved. The total chromatographic time was approximately ten minutes. The peak symmetry was excellent.

Separation 5 (Fig. 18) produced one significant difference from that of separation 4. The kappa value for component 5 is reduced from 7.9 to 3.85. This results in a reduction in the total chromatographic time to approximately six minutes. Apart from OTC and TC, excellent resolution and peak symmetry was achieved.

Separation 6 (Fig. 19) produced a very minor reversal of elution order for OTC and TC, but in the mixture chromatogram the two

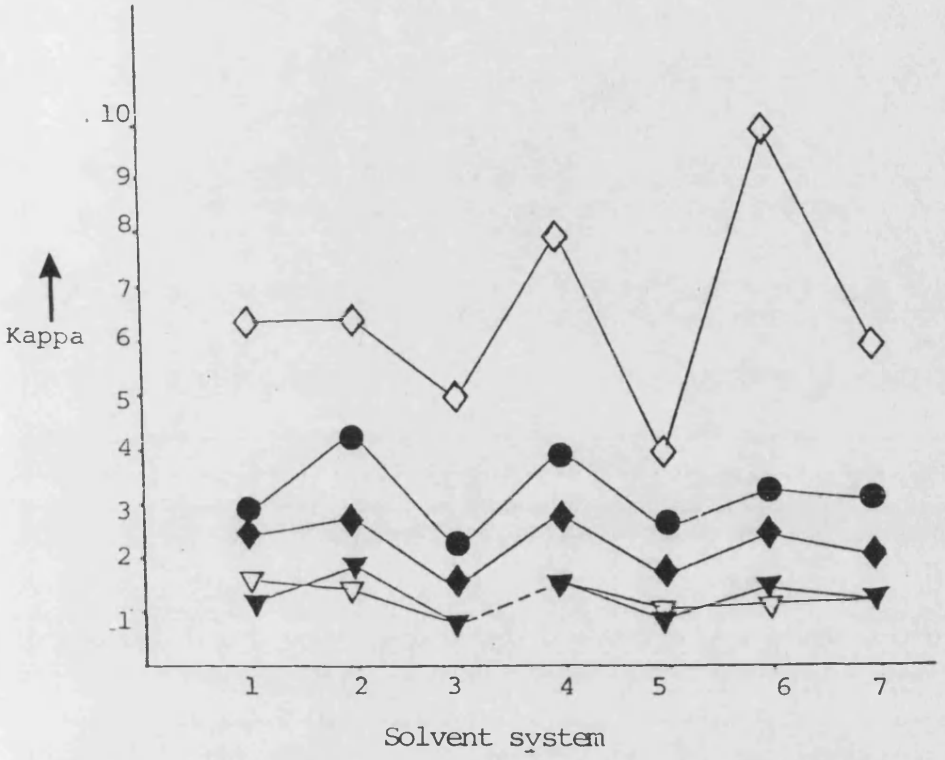
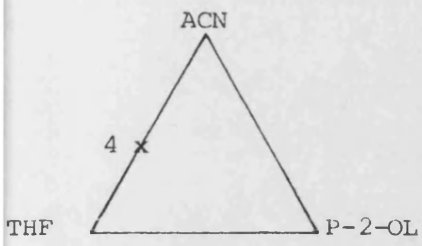


Fig. 16 Elution order of tetracyclines with different solvent systems.

▼ OTC
▽ TC
◆ demethyl CTC
● methacycline
◇ minocycline



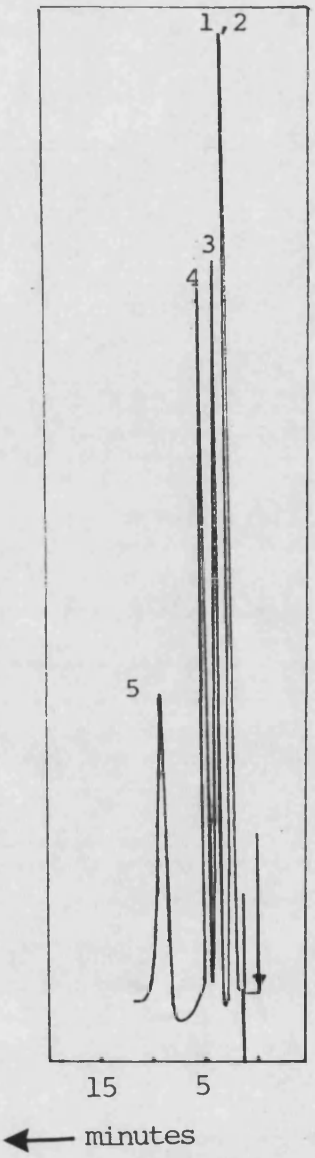
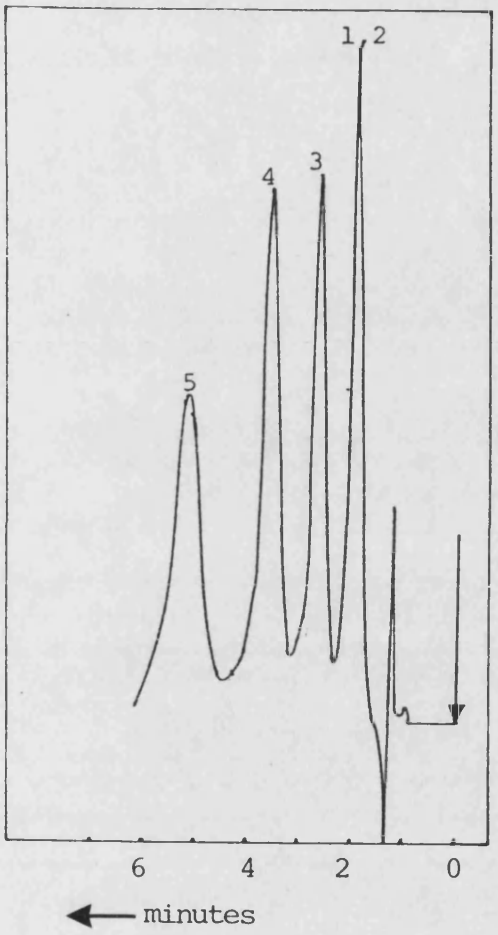


Fig.17. Isocratic separation of tetracyclines with solvent system 4.

Peak identiy	Operating conditions	
1. OTC	Column PRP-1	25cm
2. TC	Mobile phase:-	
3. demethyl CTC	Propan-2-ol	0%
4. methacycline	THF	7.1%
5. minocycline	ACN	10%
	Buffer	81.9%
	Temperature	42°C



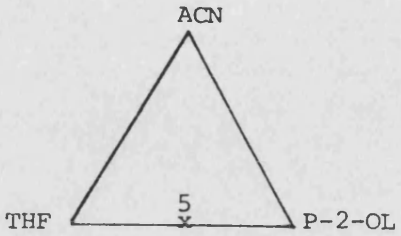


Fig. 18 Isocratic separation of tetracyclines with solvent system 5

Peak identity	Operating conditions:-	
1. OTC	Column	PRP-1 25cm
2. TC	Mobile phase:-	
3. demethyl CTC	Propan-2-ol	11%
4. methacycline	THF	7.1%
5. minocycline	ACN	0%
	Buffer	81.9%
	Flow rate	1.1 ml/min
	Temperature	42 °C

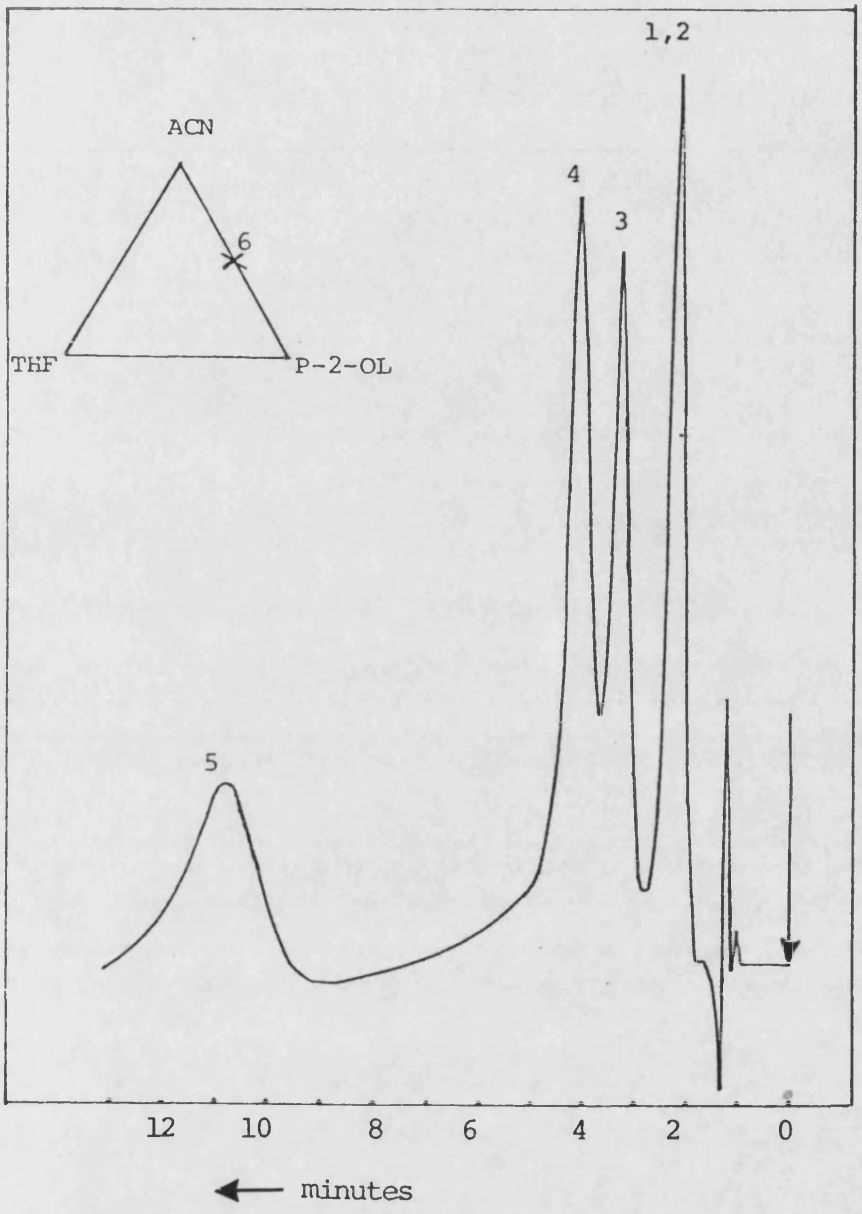


Fig. 19 Isocratic separation of tetracyclines with solvent system 6.

Peak identity	Operating conditions:-	
1. OTC	Column PRP-1 25cm	
2. TC	Mobile phase:-	
3. demethyl CTC	Propan-2-ol	11%
4. methacycline	THF	0%
5. minocycline	ACN	10%
	Buffer	79%
	Flow rate 1.1 ml/min	
	Temperature 42 °C	

components eluted together. The kappa value for component 5 was increased to 9.85. The resolution between demethyl CTC and methacycline was approximately 60%. Clearly separation 6 was not as favourable as separations 4 and 5.

Separation 7 (Fig. 20) produced reduction in kappa value of minocycline and co-elution of OTC and TC. The resolution was as good as separation 5, but the total chromatographic time was eight minutes.

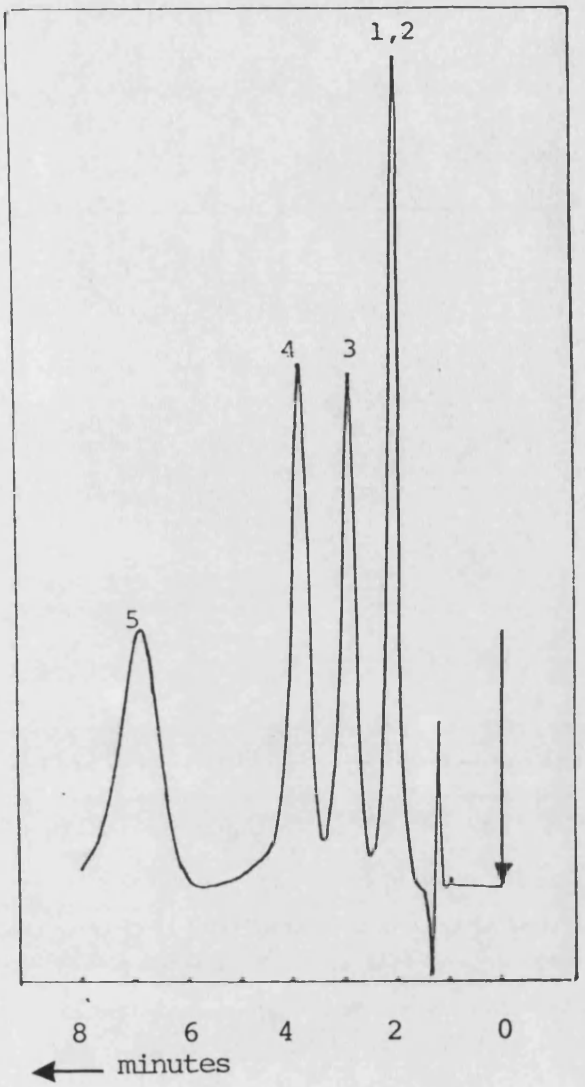
CONCLUSIONS

After examination of the seven chromatographic separations the following points may be made:-

1) One organic modifier alone is not sufficent to effect good resolution of the test mixture.
2) The best resuts were obtained with solvent system 5, which produced excellent resolution and peak symmetry in the shortest possible time (6 min). Separations 4 and 7 are also worth considering if overall time taken is not an issue.

pH PROFILE OF SEPARATION 5

Having established that the solvent system 5 was the optimum system to separate the tetracyclines, it was decided to carry out a pH profile study of the system. The buffer was prepared as before, but



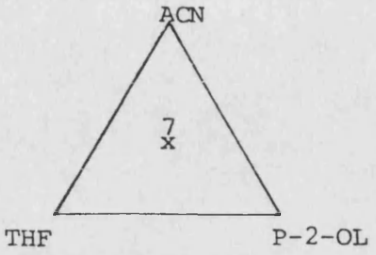


Fig.20 Isocratic separation of tetracyclines with solvent system 7

Peak identity	Operating conditions:-	
1. OTC	Column	PRP-1 25cm
2. TC	Mobile phase:-	
3. demethyl CTC	Propan-2-ol	6.6%
4. methacycline	THF	4.26%
5. minocycline	ACN	6%
	Buffer	83.1 %
	Flow rate	1.1 ml/min
	Temperature	42 °C

the volume of 0.1M HCl was varied to achieve different pH values. Table 37 shows all the different pH values and the corresponding kappa values.

Table: 37 Influence of pH upon retention

x ml 0.1N HCl	pH	OTC/TC	demethyl-CTC	methacycline	minocycline
60 ml	3.23	1.38	2.50	4.95	1.0
55	4.03	1.44	2.44	3.94	2.16
50	5.10	1.38/1.22	2.28	3.39	4.50
47	5.70	1.33	2.22	3.27	5.00
44	6.20	1.33	2.11	3.22	5.17
40	6.9	1.11	1.72	2.83	4.89

A plot of kappa values vs pH is shown in Fig. 21

Examination of the chromatograms reveal that the best separation is achieved in the pH range 5.7-6.2. A typical chromatogram at pH 5.7 is shown in Fig. 22.

At this point it would be convenient to explain why the tetracyclines elute in the order that they do. The structural formulas of the tetracyclines are shown on page 248.

The structures of OTC and TC are very similar. Due to the presence of the C_5OH in OTC, the log P value of the molecule is reduced

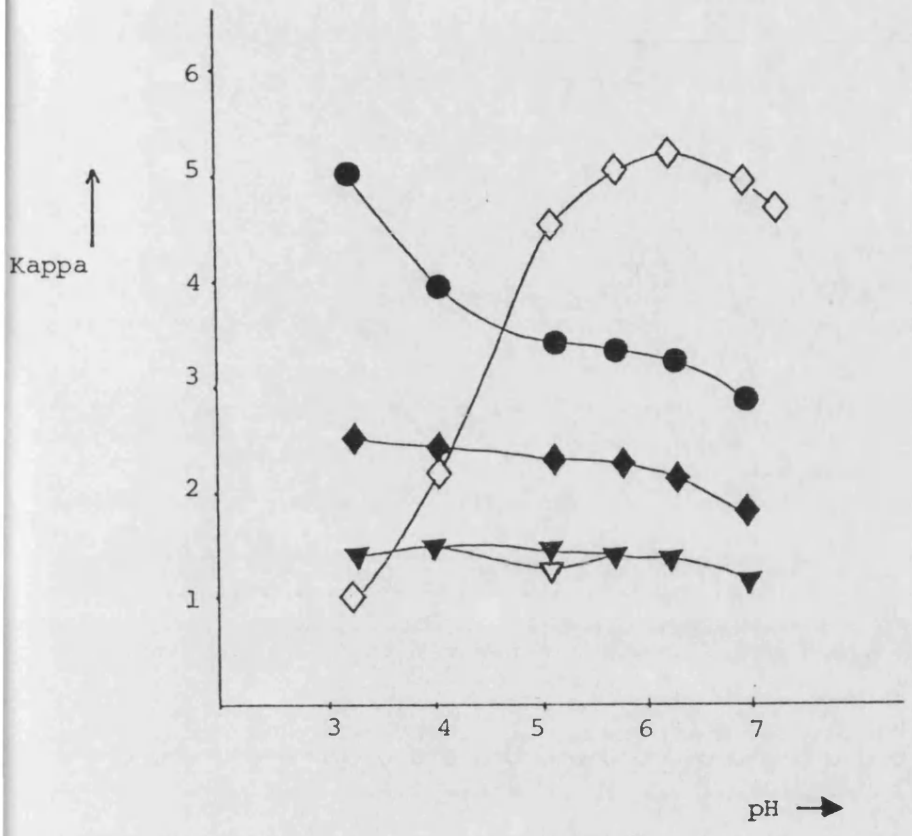


Fig. 21 pH profile of tetracyclines with solvent system 5

▼ OTC

▽ TC

◆ demethyl CTC

● methacycline

◇ minocycline

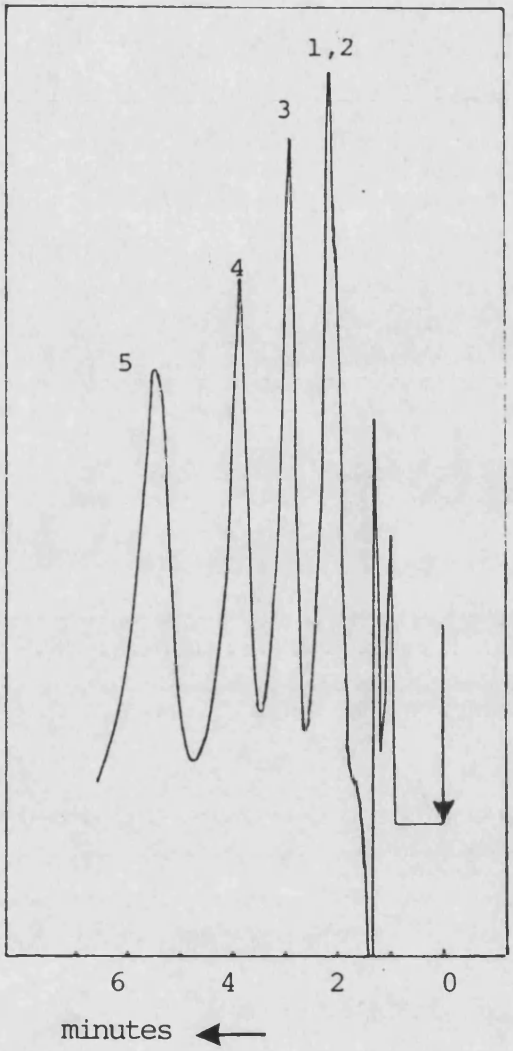
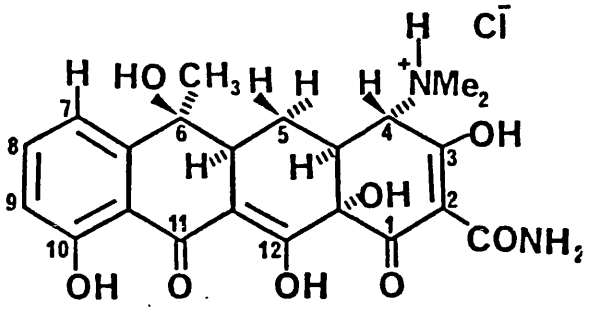
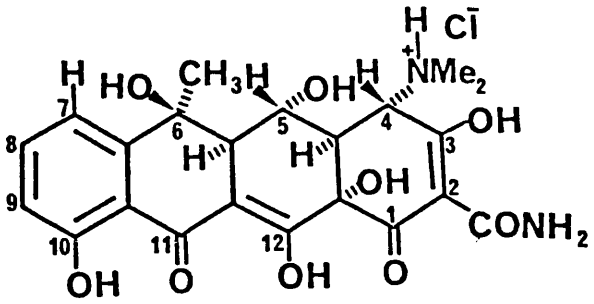


Fig. 22 Isocratic separation of tetracyclines with solvent system 5. The pH of the buffer is 5.7. Operating conditions as in Fig. 18.

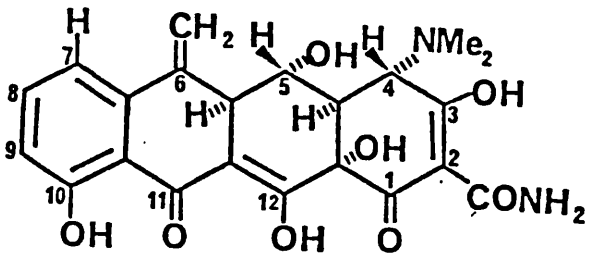
1 OTC
2 TC
3 demethyl CTC
4 methacycline
5 minocycline



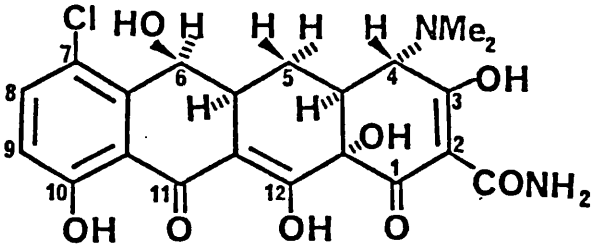
Tetracycline



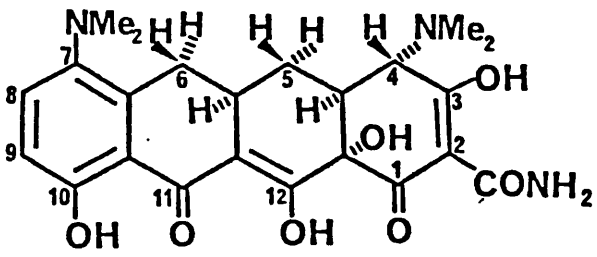
Oxytetracycline



Methacycline



6-demethyl chlortetracycline



Minocin

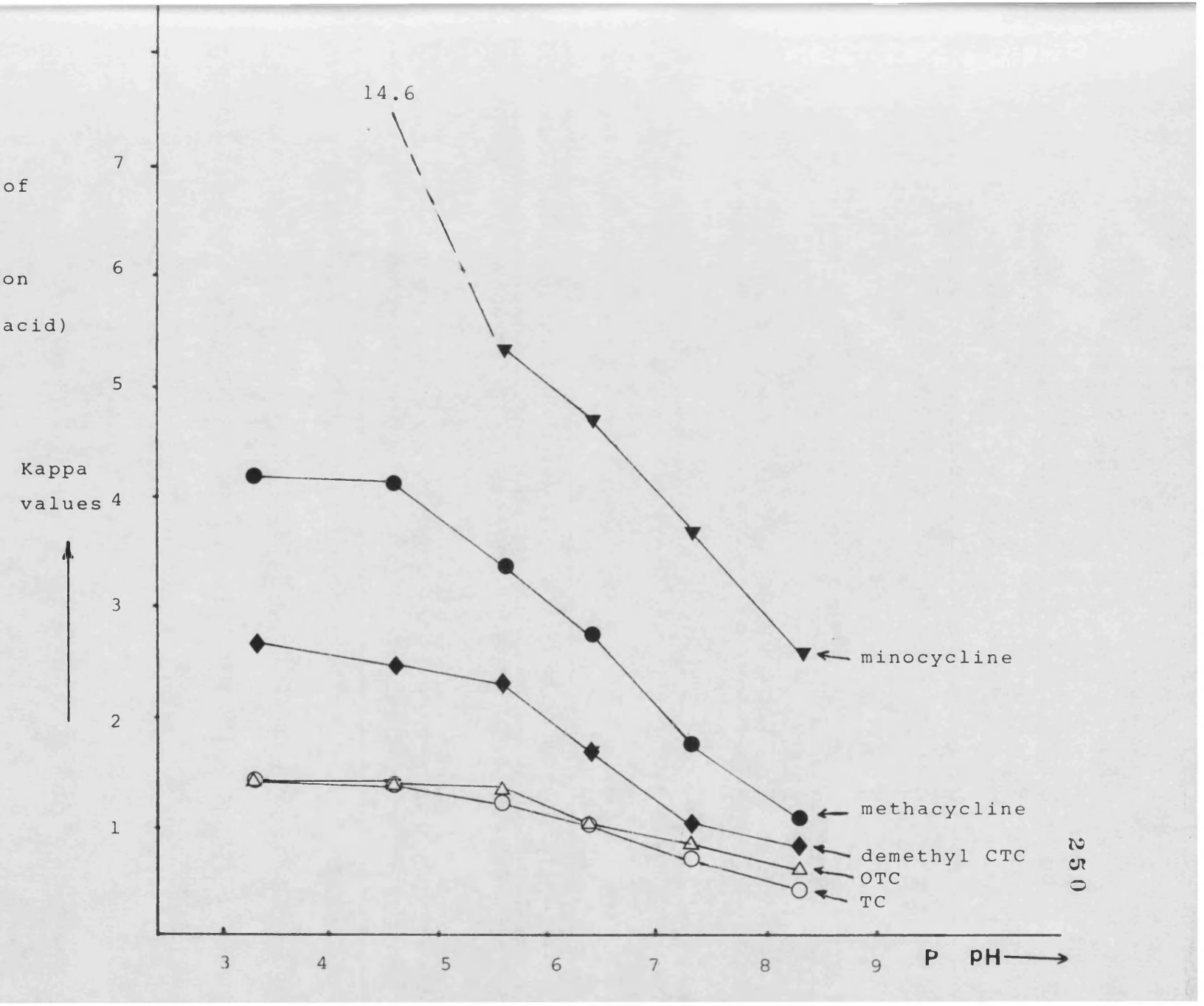
which should result in a decrease in retention. Since OTC and TC elute together immediately after the solvent front the percentage of organic modifier in the mobile phase needs to be reduced before a separation is possible.

In demethyl CTC, Cl is substituted on the aromatic ring and adds almost one log P value to the hydrophobic fragmental constant. This would result in an increase in retention but the effect is very much reduced due to loss of the C_6 CH_3 whic has a hydrophobic fragmental constant of 0.7.

In methacycline, the loss of OH at C_6 results in more retention due to greater hydrophobicity. Addition of non-polar $=CH_2$ also helps to increase retention.

In minocycline, the presence of two CH_3 on the nitrogen at C_7 increases retention, also aided by the absence of OH groups from C_5 and C_6. Reducing the pH (Fig. 21) results in a dramatic fall in the kappa values for minocycline. This is because as the pH is lowered the C_7 NMe_2 group becomes increasingly ionised . This hypothesis was tested using a negative ion-pairing reagent, 1-heptanesulphonic acid (HSA) at a concentration of 5 mM. The kappa values of OTC, TC, demethyl CTC and methacycline behave similarly as the pH is lowered (Fig. 23), but the kappa values for minocycline rose dramatically at pH 5.5. This indicates that ion-pair formation was taking place in the system. Ion pairing also occurs at the C_4 NMe_2 which is common to all the tetracyclines, but due to the presence of two NMe_2

Fig. 23. pH profiles of tetracyclines in the presence of pairing ion (1-Heptane sulphonic acid)



on minocycline, this compound is retained much more than the other tetracyclines.

SUMMARY

The use of four solvents has been demonstrated to accomplish optimization of resolution in HPLC. The four solvents used for the development of a reversed phase isocratic separation of five tetracyclines were acetonitrile, tetrahydofuran, propan-2-ol and buffer. The series of seven chromatograms, as defined by the optimization routine, were carried out on the tetracycline mixture. One solvent system was chosen after qualitative examination of chromatograms. The pH profile study was carried out on the optimum solvent system and arguments presented in favour of the elution order of tetracyclines. The pH profile study demonstrated that no further improvement in selectivity or time of analysis could be obtained by changing the optimised solvent pH (5.0) to another pH within the range 3 and 8. The pH study provided clear reasons for the elution order of the test mixture components.

Section 2

Investigation into the retention mechanisms involved between tetracyclines and PRP-1 material

Experimental

Setting up the resin

Preparation of tetrapropyl ammonium fluoride

Results and discussions

Effect of pairing-ion and competing anion concentration

Effect of ion-pair anions

Stereochemical effects on retention

Adsorption

Discussion and conlcusions

INVESTIGATION INTO THE RETENTION MECHANISMS INVOLVED BETWEEN TETRACYCINES AND PRP-1 MATERIAL

After development of the analytical system on the PRP-1 material, it was decided to determine the types of interactions which take place between tetracyclines and the polymer PRP-1. Knox and Jurand (1979), working with SAS-hypersil concluded that under mild acidic conditions (pH 3-5) the predominant mode of retention was the formation of the zwitter ion-pairs between the zwitter ionic forms of TC and the adsorbed zwitter ions of EDTA. The general performance was said to be further improved by the addition of salts acting as counter-ions. The addition of hydrophobic counter-ions to the mobile phase to enhance retention and resolution has been widely used in the HPLC of charged organic species on alkyl-modified silica (Bidlingmeyer 1980; Tomlinson et al. 1978).

Other studies have focused on identifying the interactions that occur in the presence of the counter-ions, and several mechanisms have been proposed (Knox and Jurand, 1976; Scott and Kucera 1979). The ion-pair mechanism is one where it is suggested that ion-pairs form between analyte ion and the counter-ion prior to sorption on a hydrophobic alkyl-modified silica stationary phase. Other workers suggested an ion-exchange mechanism, where the counter-ions are first sorbed and these charged sites serve as exchange sites for the analyte ions. It has also been suggested that both occur and the extent to which one is more significant than the other is a function

of the hydrophobicity of the pairing ion. If very long-chained, bulky, hydrophobic counterions are used, micelle formation and molecular size factors are significant parameters that influence retention. Cantwell and Puon (1979) proposed a double layer model that accounts for retention of organic ions onto Amberlite XAD-2, a poly(styrene-divinyl benzene) co-polymers which acts as a hydrophobic adsorbent in LC applications. In this model, the analyte cation (or anion) is sorbed onto the XAD-2 surface as a primary layer, and small counter-ions such as Cl^- (or Na^+) occupy the diffuse layer. Iskandarani and Pletrzyk (1982) studied the effect of tetraalkylammonium salts, inorganic co-anions, mixed solvents, added inert electrolytes and pH on the retention of anions derived from organic acids on PRP-1. A retention model was proposed which took into account the major equilibria that influence the retention of an organic analyte anion on PRP-1 under the specified experimental conditions.

In this report, the main emphasis would be to explore as widely as possible, the factors which influence the retention of tetracyclines on PRP-1, and relate them to some entity, such as hydrophobic parameters, M_R(alkyl), M_R(anion) etc. Statistical analysis of the data will also be carried out.

EXPERIMENTAL

SETTING UP THE RESIN

The ion-exchange resin IRA 400 was used to change the nature of the anions of the pairing ions. A similar procedure was followed for all the transformations as follows:-

A slury of the resin was made in 1 N HCl and packed into a glass chromatographic column. The resin was washed with distilled water until the filtrate was neutral. The resin is now charged with Cl^- ions. Tetrapropylammonium bromide (TPABr) was dissolved in distilled de-ionised water and added to the column and tetrapropyl-ammonium chloride (TPACl) collected. The column was washed with more distilled water to ensure complete removal of TPACl. The eluates were combined, evaporated almost to dryness and freeze-dried overnight. All the conversions, except tetrapropylammonium fluoride (TPAF), were carried out in a similar manner. The resin was first charged with the desired ion, then the substrate solution in distilled water was added to the column in order to exchange ions.

PREPARATION OF TETRAPROPYL AMMONIUM FLUORIDE (TPAF)

Stoichiometric amounts of TPABr and sodium fluoride (Na F) were mixed in methanol (300ml) and stirred at room temperature for 24Hrs. A precipitate was formed which gradually changed colour to light grey.

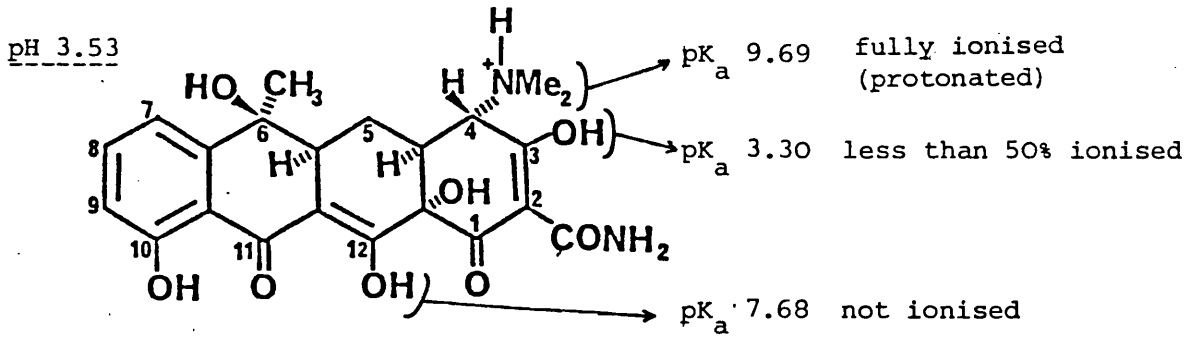
The mixture was filtered, and the filtrate was evaporated to dryness.

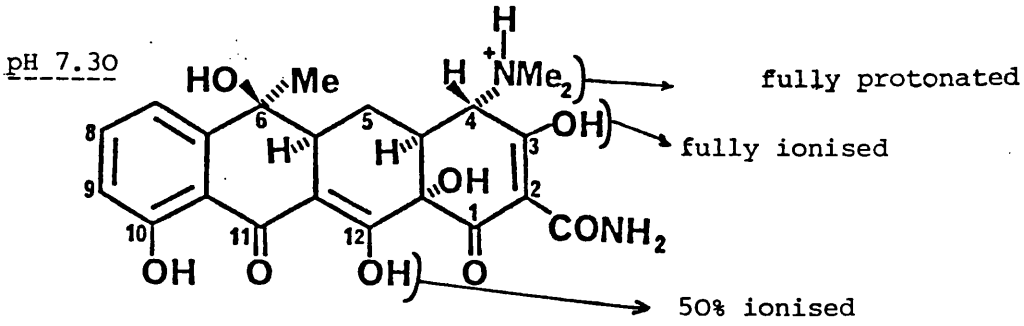
The powder (TPAF) was white and crystalline.

RESULTS AND DISCUSSION

The tetracycline molecule has three well-documented ionisation stages with pKa values of 3.3, 7.68 and 9.69, as illustrated in Fig. 24

Fig: 24 The ionisation pattern of tetracycline hydrochloride at pH 3.53 and 7.3





Therefore, at pH 7.3 the tetarcycline molecule is predominantly an anion. The chromatographic system may be considered to consist of the following components:-

PRP-1 material, the alkyl-ammonium salt and anionic analyte.

EFFECT OF PAIRING ION AND COMPETING ANION CONCENTRATION

If the chromatographic retention mechanism is due to the pairing-ion (alkyl-ammonium salt) being adsorbed on to the stationary phase (PRP-1), thus forming an in-situ ion-exchange surface as is the case for alkyl-bonded silica materials (Bidlingmeyer, 1980; Tomlinson et al. 1978), then:

a) An increase in the concentration of alkyl-ammonium pairing-ions should increase the retention of negatively-charged tetracyclines.

b) An increase in the concentration of competing anions (Cl,Br,F) should decrease the retention of anionic form of the tetracyclines.

Concentrations of TPABr ranging from 1 to 20mM were equilibrated on the chromatographic column and the retention values for the tetracyclines determined to test (a) above. Retention was found to be dependent upon pairing-ion concentration, reaching a maximum at 5mM. This concentration was chosen to test (b) above, using sodium salts of acetate, nitrate and sulphate as competing counter-ions. The results are listed in Table. 38

The kappa values for OTC and TC are almost identical (0.8-0.9). Since the two compounds have capacity ratios of less than 1.0, the two peaks were not retained adequately to determine the effect of the pairing-ion, therefore the kappa values for OTC and TC were disregarded.

Statistical analysis (t test) was carried out on the kappa values of demethyl CTC, methacycline and minocycline. The following points were deduced from the t-test.

1) The capacity-ratios decreased significantly as the concentration of the counter-ion was increased (A1,A4; B1,B4; C1,C4).

2) There was a significant difference between the effects of the different counter-ions, at a concentration of 0.2M, the order being NO_3^- > SO^{2-}_4 > acetate$^-$ (A4,B4; A4,C4; B4,C4).

kappa value increase ⟶

Table 38. The effect of counter-ion concentration on kappa values

[$NaNO_3$] mM	demethyl-CTC	methacycline	minocycline	
0.0	1.45	2.5	4.6	..... A-1
0.05	1.0	1.6	3.1	..... A-2
0.1	1.0	1.4	2.9	..... A-3
0.2	1.0	1.3	2.9	..... A-4

[Na acetate] mM	demethyl-CTC	methacycline	minocycline	
0.0	1.45	2.5	4.6	..... B-1
0.05	1.0	1.3	3.0	..... B-2
0.1	1.2	2.0	3.8	..... B-3
0.2	1.2	1.9	3.5	..... B-4

[Na_2SO_4] mM	demethyl-CTC	methacycline	minocycline	
0.0	1.45	2.5	4.6	..... C-1
0.05	1.2	2.0	3.8	..... C-2
0.1	1.2	1.9	3.8	..... C-3
0.2	1.2	1.8	3.8	..... C-4

EFFECT OF ION-PAIR ANIONS

TPABr was converted to TPACl and TPAacetate, using the IRA-400 resin, as indicated in the experimental section. TPAF was prepared chemically. The kappa values obtained with these pairing-ions are listed in Table 39.

Table 39 The kappa values with various ion-pairing reagents (5mM)

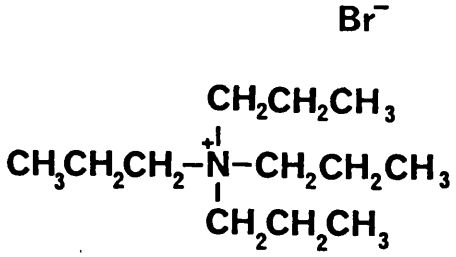
	OTC	TC	demethyl CTC	methacycline	minocycline	
TPAF	0.835	0.855	1.21	1.875	3.725	...D
TPACl	1.083	0.875	1.35	2.000	4.125	...E
TPA acetate	1.000	0.900	1.60	2.4000	4.300	...F
TPABr	1.000	1.200	1.55	2.5500	4.550	...G

Statistical analysis (t-test) proved that significantly different kappa values were being obtained by using the different ion-pairing reagents. Since, all the variables were kept constant, the change in kappa values must be due to the negative part of the pairing-ion competing with the negatively charged tetracyclines for retention by the positively-charged tetrapropyl ammonium ion. The fluoride ion was most effective in reducing retention of the tetracyclines, followed by chloride, acetate and bromide respectively. For the halogen series, the unit charge per surface area is highest for fluoride and decreases for chloride and then bromide. This suggests

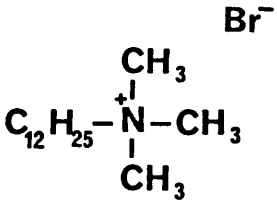
that fluoride is the most effective counter-ion because it is able to approach most closely to the positively-charged tetrapropyl ammonium ion. This is the competitive mechanism considered to occur in ion-exchange chromatography.

STEREOCHEMICAL EFFECTS ON RETENTION

The nitrogen atom in TPABr is surrounded by four bulky alkyl groups, whereas in dodecyl trimethylammonium bromide (DTMABr), the nitrogen is much more exposed.



TPABr



DTMABr

DTMACl was also tested on the column (prepared using IRA-400 resin). The kappa values are listed in Table 40.

As can be seen from Table 40 longer kappa values were obtained with DTMABr than TPABr and with DTMACl compared to TPACl. This was to be

expected due to the larger hydrocarbon content of the former (15C compared to 12C). Hence, significantly different kappa values between G-H and E-J.

Table 40 The kappa values of tetracyclines using various pairing-ions (5mM).

	OTC	TC	demethyl-CTC	metha-cycline	minocycline	
TPABr	1.000	1.20	1.55	2.55	4.55	...G
DTMABr	2.220	2.33	5.45	5.11	8.56	...H
TPACl	0.875	1.08	1.34	2.00	4.13	...E
DTMACl	2.340	2.45	5.33	5.00	8.55	...J

But values for DTMABr and DTMACl were almost identical. Further work was carried out using a homologous series of the tetraalkyl ammonium compounds i.e.

Tetraethyl ammonium bromide TEABr
" " chloride TEACl
Tetramethyl ammonium bromide TMABr
" " chloride TMACl

These pairing-ions were loaded on to the column at concentrations of 5mM. The kappa values are listed in Table 41.

As can be seen from the Table 41 the kappa values for TMA are significantly different (t-test) from TEA pairing-ion. The effect of Cl or Br ions may be distinguished as follows:-

TMABr and TMACl	Kappa values	Cl > Br	...P
TEABr and TEACl	kappa values	Br > Cl	...Q
TPABr and TPACl	kappa values	Br > Cl	...R
DTMABr and DTMACl	kappa values	almost similar	...S

Table 41. Kappa values with different pairing-ions

	OTC	TC	demethyl-CTC	metha-cycline	minocycline	
TMABr	.75	1.0	1.25	1.88	3.69	..... K
TMACl	1.13	1.25	1.88	2.75	4.75	 L
TEABr	1.07	1.13	1.75	2.69	4.75	..... M
TEACl	1.0	1.125	1.5	2.375	4.5	..... N
TPABr	1.0	1.2	1.55	2.55	4.55	..... G
TPACl	.875	1.08	1.34	2.0	4.13	..... E
DTMABr	2.22	2.33	5.45	5.11	8.56	..... H
DTMACl	2.34	2.45	5.33	5.0	8.55	..... J

Therefore, when the nitrogen atom of the pairing-ion is readilly accessible, there is little or no difference between the kappa values

of chloride or bromide salts of the pairing-ions (P and S). But when the nitrogen atom is less accessible, the smaller ion i.e. Cl^- is more effective in reducing retention (Q and R).

The kappa values with different pairing-ions are listed in Table 42 Also included are relavant parameters e.g. M_R alkyl, M_R anion etc., where M_R stands for molar refraction.

Single and multiple regressions were carried out on the data in Table 42 in order to determine the variable parameter which influences retention of the five tetracyclines. But no significant correlation results were obtained. This indicates that although the variables already discussed effect retention to some extent, the dominant mechanism may be different.

ADSORPTION

Since the PRP-1 packing material is poly(styrene-divinylbenzene) polymer, it seems probable that the concept of adsorption would play a major part in retention. Hence the changes in hydrophobicity ( ) of the tetracycline molecules should be considered. log P

To study this concept, an aliquot (50 ml) of the buffer, without the pairing-ion and the organic modifier, was mixed with vairing amounts

	M_R alkyl	M_R anion	OTC	TC	DMCTC	METHA	MINOCIN	alkyl	Mol. Wt. (alkyl)	Mol. Wt. (total)	Mol. Wt. (anion)
TMA Cl	5.65	6.03	1.13	1.25	1.88	2.75	4.75	0.56	15	109.5	35.5
TMA Br	5.65	8.88	0.75	1.0	1.25	1.88	3.69	0.56	15	153.9	79.9
TEA Cl	10.3	6.03	1.0	1.125	1.5	2.375	4.50	1.02	29	165.5	35.5
TEA Br	10.3	8.88	1.07	1.13	1.75	2.69	4.75	1.02	29	209.9	79.9
TPA Cl	14.96	6.03	1.083	0.875	1.344	2.0	4.125	1.55	43	221.5	35.5
TPA Br	14.96	8.88	1.0	1.2	1.55	2.55	4.55	1.55	43	265.9	79.9
TPA F	14.96	0.92	1.0	1.2	1.55	2.55	4.55	1.55	43	204.99	18.99

Table 42. The kappa values for different tetracyclines using various ion-pairing reagents. The hydrophobicity parameters (M_R) are also included along with other variables e.g. molecular weights.

DMCTC = 6-demethyl CTC

METHA = methacycline

MINOCIN = minocycline

of PRP-1 material (see Table 43). A similar solution was prepared which also contained the pairing-ion (TEA Br at 2 mM concentration). The flasks were placed in a mechanical shaker at 25°C for 24 hrs. The mixtures were allowed to settle and the UV absorbance of the aqueous phase was recorded (Table 43). The changes in absorbance vs concentartion of the packing material is recorded in Fig. 25 .

In the presence of 200 mg PRP-1 the adsorption of the drug was almost double that in the presence of 100 mg PRP-1, indicating that adsorption is directly proportional to the amount of PRP-1. At 300 mg PRP-1 the adsorption was only slightly greater than at 200 mg, indicating insufficent drug in the aqueous phase to saturate the PRP-1 packing material.

When the pairing-ion (TEA Br) was included in the above system, the adsorption was increased, but only by 31 and 25 % respectively for the 100 mg and 200 mg PRP-1. This strongly suggests that the major retention mechanism is adsorption of the tetracyclines to the PRP-1 material. The average increase in retention with TEA Br was 28 %

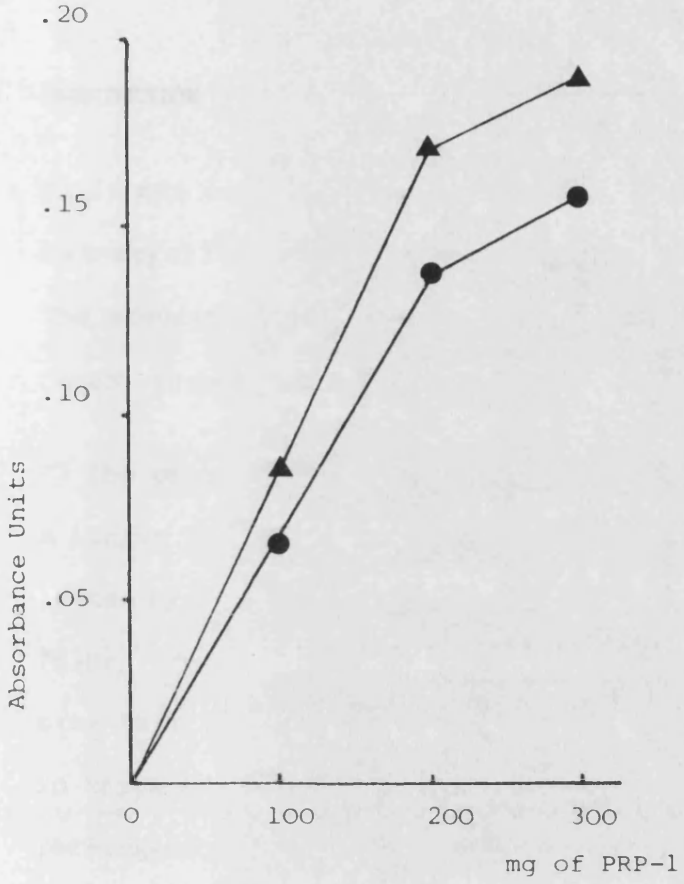


Fig. 25. Adsorption of "drug" by PRP-1 from an aqueous solution of constant concentration. (Absorbance units representing amount of drug removed from the aqueous buffer).

▲

●

DISCUSSION AND CONCLUSIONS

1) It can be concluded that the main mechanism of retention of the tetracyclines on PRP-1 packing material is by simple adsorption. The adsorption is further increased in the presence of pairing-ions (a 28% increase resulted by using TEA Br)

2) The pairing-ions with a higher log P value than TEA Br (i.e. with a longer hydrocarbon chain) e.g. TPA Br, DTMA Br etc. would adsorb on to the hydrophobic packing material at a greater concentration than TEABr, and therefore would increase retention of tetracyclines even more than TEA Br. This is best seen in Fig. 26 , where an increase in kappa value is observed with increase in the carbon content of the pairing-ions. Accesssibility of the charged nitrogen on the pairing-ion is also very important in deteriming retention, as indicated by incresed kappa values obtained with DTMA Br than TPA Br.

3) The presence of counter-ions decreased kappa values, the order of influence being F > Cl > Acetate > Br

4) Retention may be further reduced by including competing electrolytes (anions) in the chromatographic system.

Table 43 Absorbance values with and without PRP-1 and pairing-ion

Without pairing-ion		With pairing-ion	
	100mg PRP-1		
Buffer + drug*	= 0.520	Buffer + drug + P-ion	= 0.515
Buffer + drug + PRP-1	= 0.455	Buffer + drug + P-ion + PRP-1	= 0.430
	200mg PRP-1		
Buffer + drug	= 0.530	Buffer + drug + P-ion	= 0.527
Buffer + drug + PRP-1	= 0.392	Buffer + drug + P-ion + PRP-1	= 0.355
	300mg PRP-1		
Buffer + drug	= 0.525	Buffer + drug + P-ion	= 0.520
Buffer + drug + PRP-1	= 0.367	Buffer + drug + P-ion + PRP-1	= 0.330

(* drug used is minocycline)

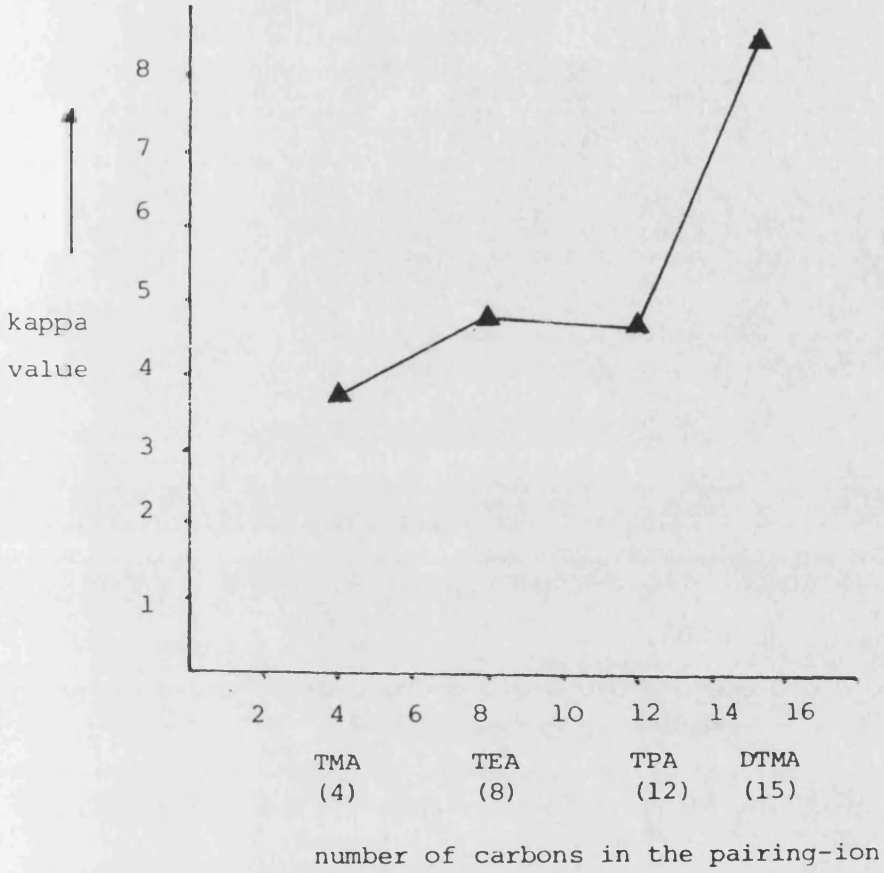


Fig. 26 Change in kappa value with the number of carbons in the pairing-ions. Only bromide salts are represented.

SECTION 3

Determination of the rate constants for the degradation of TC and OTC.

Instrumentation

Results and discussions

Kinetics of TC and its degradation products

Degradation of TC HCl into 4-epi TC HCl

Kinetics of oxytetracycline HCl (OTC HCl)

Stability of tetracycline under various physical conditions

Room temperature

Temperature $4^{\circ}C$

Heated at $60^{\circ}C$ and $100^{\circ}C$

Conclusions

DETERMINATION OF THE RATE CONSTANTS FOR THE DEGRADATION OF TC AND OTC

Rate processes are of fundamental importance to everyone connected with the pharmaceutical industry. The manufacturer must be able to show that the drug is suitably formulated to prevent the degradation of the parent compound into by-products. The medical practitioner must be assured that the drug formulation will allow the active drug to reach the intended site of action in sufficient concentration to elicit the anticipated response.

Assuming that tetracycline (TC) degrades only to anhydro TC the reaction equation would be as follows:-

$$\text{TC HCl} \underset{R_r}{\overset{R_f}{\rightleftharpoons}} \text{ATC HCl}$$

The rate of the forward reaction (R_f) is given by $R_f = -\frac{d_c}{d_t}$, where d_c is the change in concentration and d_t is the change in time. The negative sign indicates that the concentration of the drug (TC) is decreasing with time. Similarly, $R_r = -\frac{d_c}{d_t}$ indicates the rate equation for the reverse reaction.

Two studies were undertaken:-

A:- The kinetics of tetracycline and oxytetracycline were investigated under different pH conditions. Rate constants and Half life periods were calculated.

B:- The stability of a TC HCl solution was studied under different conditions of temperature, light etc.

INSTRUMENTATION

A: The mobile phase was constituted as follows:-

1) buffer at pH 5.0

2) THF at 7.1 %

3) Propan-2-ol at 11 %

The PRP-1 (15cm) column was used in this study. The drugs were weighed and dissolved in the mobile phase to give a final concentration of 0.075mg/ml. The kinetic study was carried out on the Spectra Physics SP8100 liquid chromatograph connected to an SP4200 integrator and an SP8440 UV/VIS detector operating at 272 nm, 0.08 AUFS. The flow rate was 1ml/min, the injection volume was 10 µl, and the oven temperature was 33°C. The pH of the solutions was adjusted using HCl or NaOH. The following pH values were chosen:-

TC HCl pH 1.1, 2.0, 3.4, 5.0, 7.0, 8.8, 11.6.

OTC HCl pH 1.2, 2.0, 3.0, 5.0, 6.9, 8.8, 10.0.

The prepared solutions were placed in the autosampler. Two injections at each pH value were taken on day one. Samples were subsequently injected on to the coulmn at regular intervals over a

ten day period. Either peak areas or peak heights were used to calculate the rate constants and Half life periods.

B:- The same mobile phase was used. The TC HCl solutions (0.075 mg/ml) were stored under the following conditions.

Room temperature light and dark, heated ovens at 60°C and 100°C and at a temperature of 4°C.

Injections were taken at day one and at regular intervals within a twelve day period. Only qualitative examination of the results was carried out.

KINETICS OF TETRACYCLINE AND ITS DEGRADATION PRODUCTS

RESULTS AND DISCUSSION

The tetracycline samples were analysed at a range of pH values. The degradation of TC HCl into anhydro TC was most pronounced at pH 1.1, while the epimerisation to 4-epi TC HCl took place readilly at pH 3.3. Therefore the degradation of TC HCl and anhydro TC HCl is investigated at pH 1.1, pH 3.3 is chosen for the study of epimerisation. Theresults obtained for TC HCl are listed in Table 43.

Day	t_r(min)	AREA	CONC. (M)	log(a-x)	1/(a-x)
1	2.28	193565	0.000156	---	---
2	2.37	179378	0.000145	-3.839	6896.6
3	2.36	170237	0.000137	-3.863	7299.3
4	2.31	156269	0.000126	-3.899	7936.5
6	2.36	142405	0.000115	-3.939	8695.7
8	2.39	126087	0.000102	-3.991	9803.9
9	2.42	119737	0.000097	-4.016	10362.7
10	2.40	109742	0.000089	-4.053	11299.4

Table 43.Results from kinetic study of TC HCl at pH 1.1

a= initial concentration, x= change in concentration

In order to determine whether the degration was zero order or first order etc. concentration, log(a-x) and 1/(a-x) were plotted vs. time (day). A linear relationship was achieved by plotting log(a-x) vs. time (Fig.27). Therefore the degradation of TC HCl follows a first order reaction rate. The slope was calculated to be :-

Slope = K = -0.0259

The negative sign denotes that the concentration is decreasing with time. The half life, i.e. the period of time required for a drug to decompose to one half of the original concentration, was calculated as follows:-

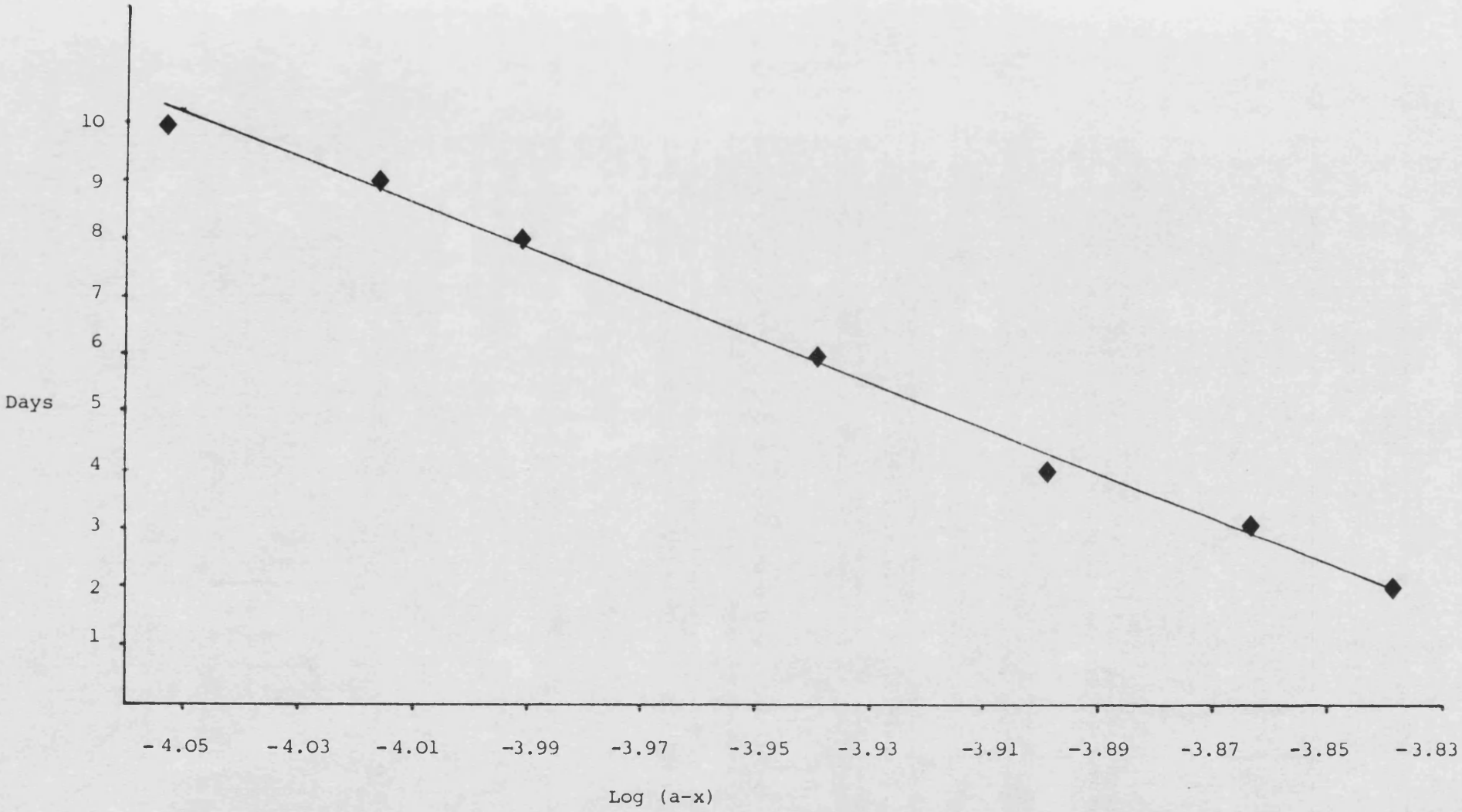
$$T_{1/2} = 0.693/K$$

$$0.693/0.0259 = 26.76 \text{ days.}$$

Single regression was carried out on the results in Table 43. The standard deviation of coefficent was low ($2.49 * 10^{-7}$), the correlation coefficient was very high (99.6%) and the F-ratio was also very high (747.99). The F-ratio means that the model of the reaction, i.e. first order, is valid.

The formation of anhydro TC was also studied at the same pH (1.1). Only peak heights were used. The data is presented in Table 44.

Fig. 27.Plot of Day vs Log(a-x) for the degradation of TC HCl. (a-x) is the change in concentration



DAY	PEAK HT. (mm)	log(a-x)	1/(a-x)
1	2.0	----	----
2	4.0	0.60206	0.2500
3	5.5	0.74040	0.1818
6	13.0	1.11400	0.0769
8	16	1.20400	0.0625
9	17.5	1.24300	0.0572
10	19.5	1.29000	0.0513

Table 44 Kinetic data for Anhydro TC at pH 1.1

Concentration (Peak ht.), log(a-x) and 1/(a-x) were plotted vs. day, but only in the first case was there a linear relatioship i.e. concentration vs. time (Fig 28). Therefore, the order of the formation of anhydro TC HCl is zero order. Although it seems odd that a drug which degrades in a First order manner should produce a product which increases in concentration at a zero order rate, this may be explained as follows:-

The model of the reaction may be as follows:-

$$\text{At pH 1.1} \quad TC \xrightarrow{K_1} ATC \xrightarrow{K_2} X$$

The main drug is degrading with a rate constant K_1, but it may also be true that ATC is degrading further into X with a rate constant K_2. Since no other peak is observed except that of anhydro TC, it may be

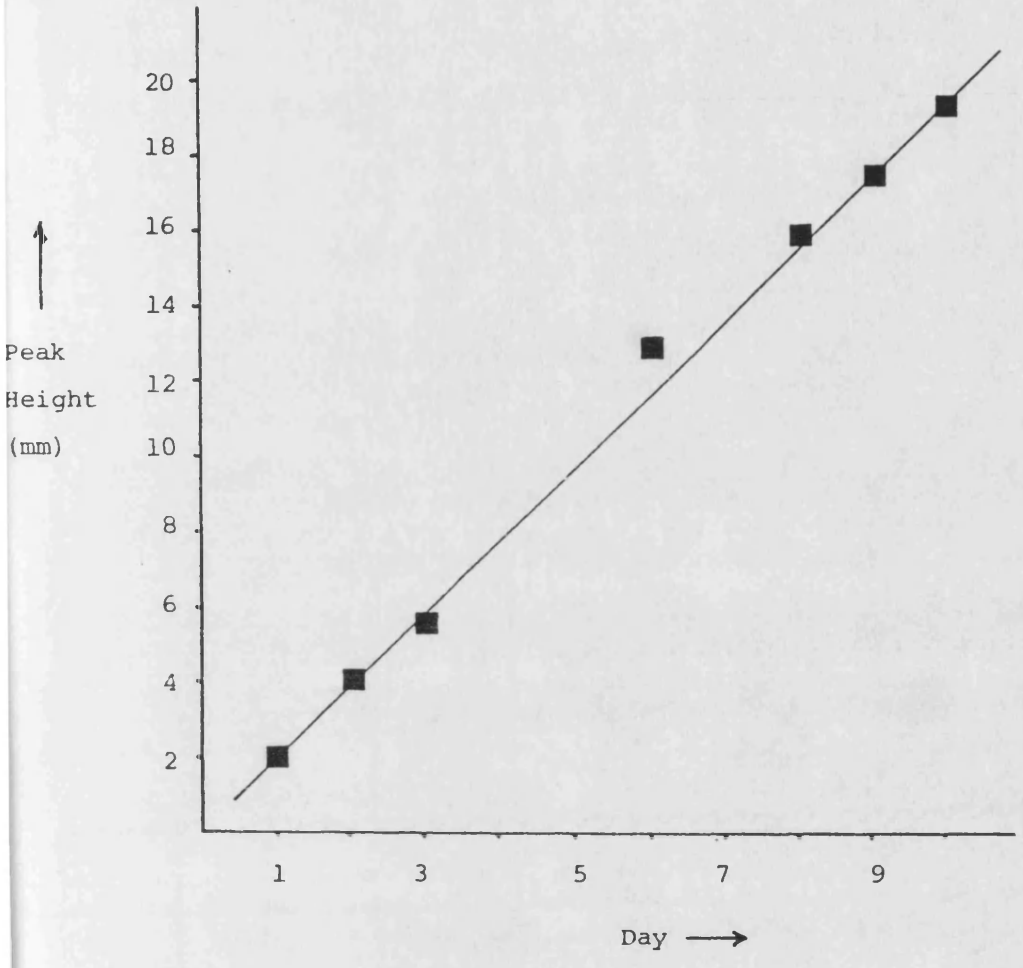


Fig 28 Plot of concentration (peak height in mm) vs day for the formation of anhydrotetracycline HCl at pH 1.1

assumed that $K_1 > K_2$. Hence, as the drug degradation results in an increase in the pool concentration of anhydro TC, a fixed proportion may be continuously removed. This results in a linear response for concentration vs. time. Furthermore, the study was carried out over a ten day period (Half life of TC = 26 days), hence only a small percentage of the drug has been degraded. This would also explain the non-appearence of other products. Even though a linear response is observed for anhydro TC in the ten day period, if the time of the experiment was to be increased to 2-3 half life periods the linear response may change.

Therefore it may be concluded that:-

1) TC degrades with a First order reaction rate.
2) AnhydroTC seems to degrade at zero order rate, but a longer time of study (2-3 $T_{1/2}$) is required.
3) Rate constant for the degradation of TC = -0.0259
4) Rate constant for the formation of anhydro TC = 1.966
5) $T_{1/2}$ for TC = 26.76 days

DEGRATION OF TC HCL INTO 4-EPI TC HCL

It had not been possible to resolve TC and 4-epi TC employing the mobile phase system used up till now. The 4-epi TC peak may be detected as a "shoulder" in the ascending part of the TC peak. But

in order to study the kinetics of the formation of the 4-epi derivative, it was necessary to resolve the two peaks. This was achieved by reducing the mobile phase strength to 25% and altering the flow rate to 0.5 ml/min.

The mobile phase was constituted as follows:
solution A: 40 ml ; solution B: 110 ml
made up to 200 ml with double distilled deionised water.
THF = 1.775 % ; propan-2-ol = 2.75 %
The pH of the buffer prior to the addition of organic modifier was 3.3. The other experimental conditions were as before.

The chromatogram of the resolution between TC and 4-epi TC is shown in Fig. 29 , and the peak area results are listed in Table 45 .

Table 45 Peak area results for the kinetic study of TC at pH 3.3

Hours	TC area	lg. area	epi-TC area	lg. epi-TC area
0	44.7948	1.65	0.7911	-0.10
19	40.8601	1.61	1.4142	0.151
44	37.3875	1.57	2.52	0.402
70	33.2748	1.52	3.54	0.549
141	25.152	1.40	10.1052	1.00
146	30.7008	1.49	13.09	1.117
192	19.953	1.30	27.54	1.44
233	16.22	1.21	54.954	1.74

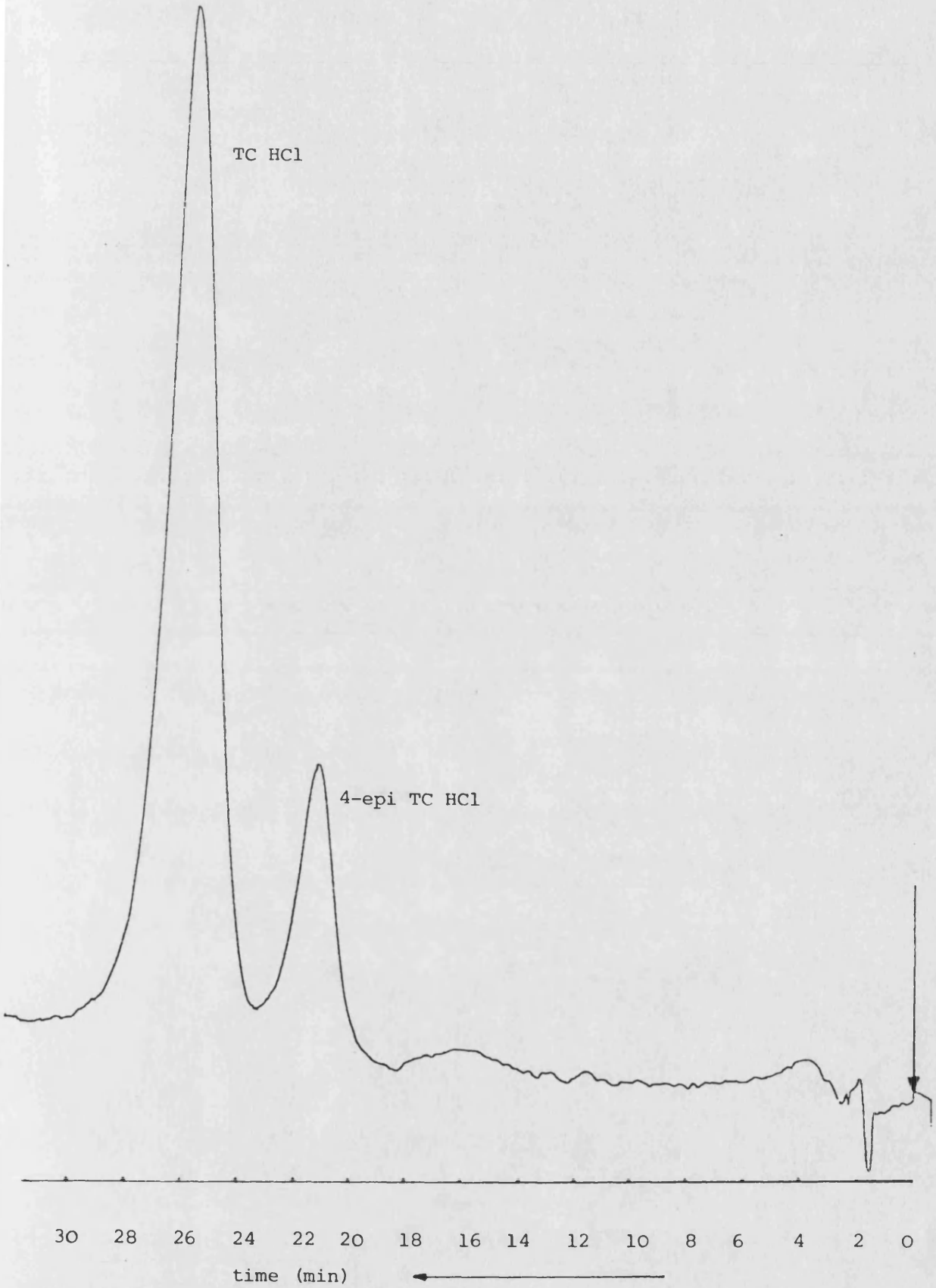


Fig 29 Resolution of TC HCl and 4-epi TC HCl using the modified mobile phase system, as outlined in the text.

A linear relationship was observed between log.(a-x) and time (see Fig 30). Therefore the formation of 4-epi TC HCl is also a first order reaction

KINETICS OF OXYTETRACYCLINE HCL (OTC HCL)

The samples of OTC HCl (at various pH values) were injected at regular intervals. From the analysis of chromatograms, the degradation of OTC HCl was pronounced at pH 5.0. the results are listed in Table 46 .

The order of reaction was determined by plotting area, log(a-x) and 1/(a-x) vs. time. A linear relatioship was observed between lg(a-x) and time (Fig. 31). Therefore, the degradation of OTC is also first order.

DAY	AREA	log(a-x)	1/(a-x)
1	134605	5.129	$7.43 * 10^{-6}$
2	126427	5.102	$7.91 * 10^{-6}$
3	117333	5.069	$8.52 * 10^{-6}$
4	99806	4.999	$1.00 * 10^{-5}$
6	87498	4.942	$1.14 * 10^{-5}$
8	73796	4.868	$1.36 * 10^{-5}$
9	69001	4.839	$1.45 * 10^{-5}$
10	63125	4.800	$1.59 * 10^{-5}$

Table 46 Kinetic data of OTC at pH 5.0 a= initial concentration x= change in concentration

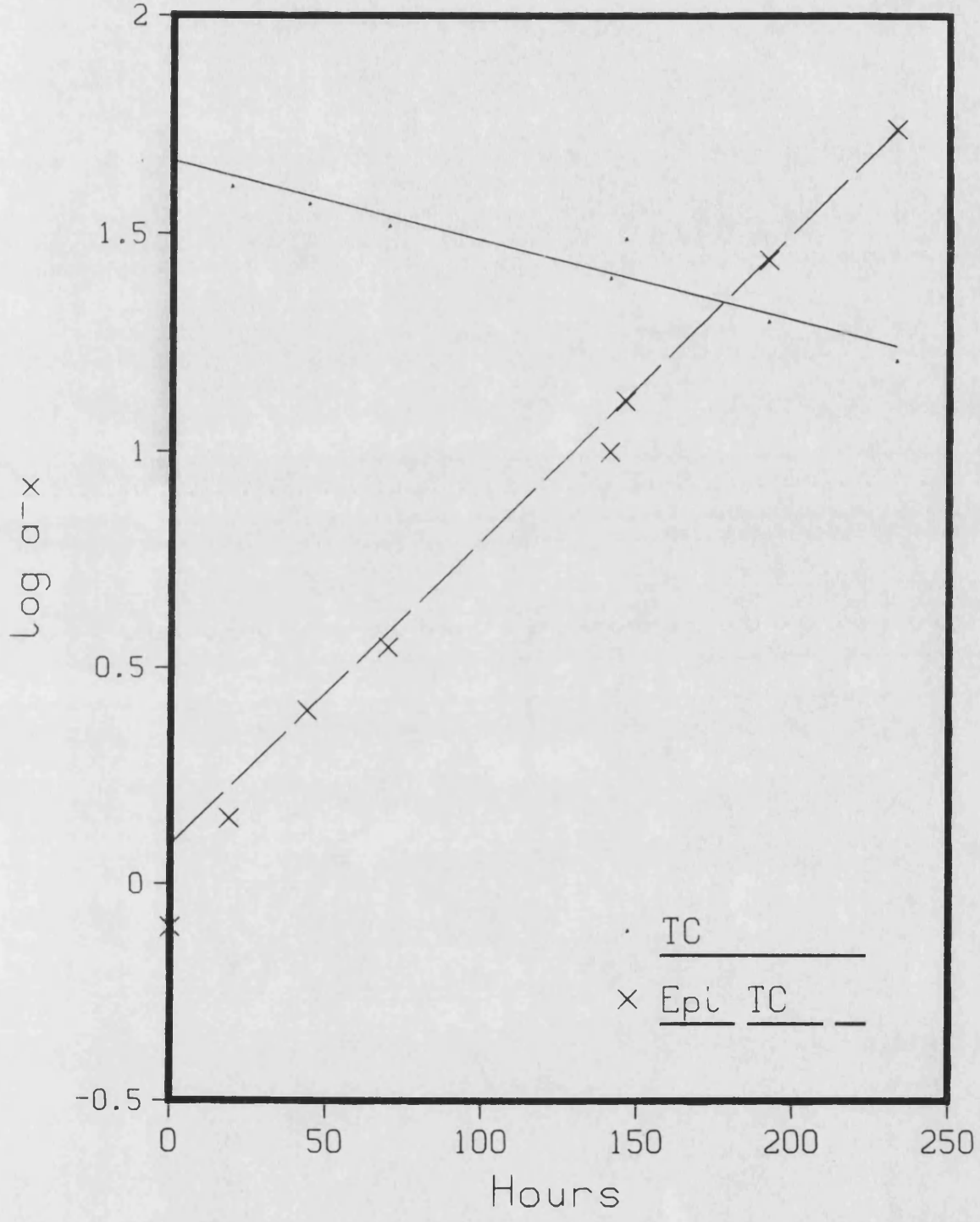


Fig. 30 A plot of log(a-x) and time for the formation of 4-epi TC HCl

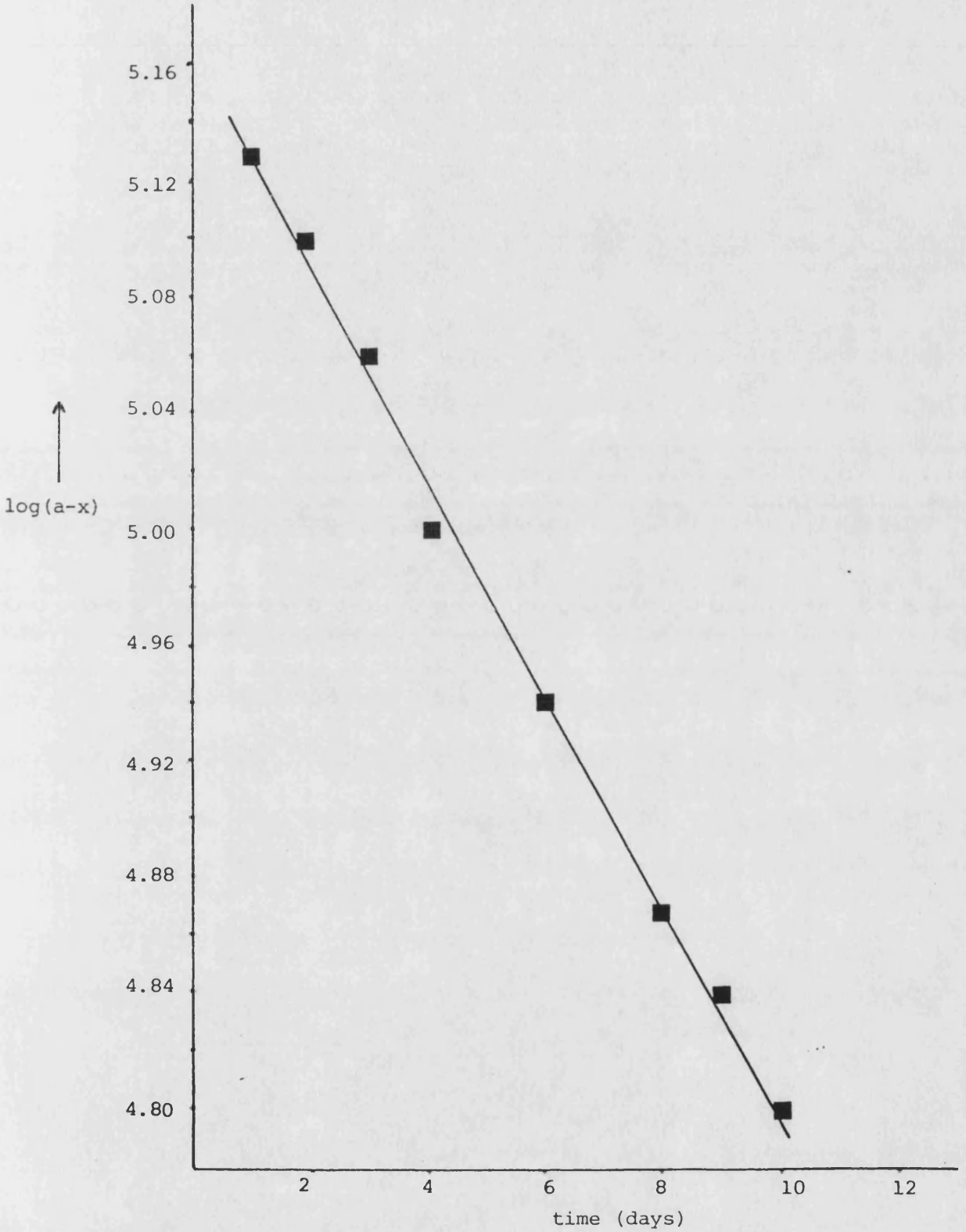


Fig. 31 Plot of log(a-x) vs Day for Oxytetracycline HCl at pH 5.0

The slope was calculated to be -0.0093

$T_{1/2}$= 0.693/k = 74.52

There was no other peak to show the presence of anhydro OTC This is because anhydro OTC is very unstable and begins to decompose as soon as it is formed. The epi-OTC peak was absent at pH 1.2 or 2.0 but present at 3.0 and 5.0, indicated as a shoulder on the OTC peak.

B:-

STABILITY OF TC UNDER VARIOUS PHYSICAL CONDITIONS

A qualitative study was also carried out on a solution of TC HCl stored under various physical conditions of temperature and light etc. The study was carried out over an eighteen day period.

ROOM TEMPERATURE SOLUTIONS KEPT IN LIGHT AND DARK

The solutions behaved fairly similarly under both the conditions. As early as day four, traces of ATC were observed in the solution kept in light. Storage in the dark did not seem to slow the degradation to any appreciable extent .

Temperature 4°C

The solution was stored in a refrigerator (3-4°C) and removed just prior to injection. A very small peak corresponding to ATC was observed on day eleven. The epi-isomer was apparent as a small shoulder in the main peak as early as day 5. No other impurities were observed.

HEAT AT 60°C AND 100°C

The tetracycline solution showed very rapid degradation at these temperatures. It was not possible to identify any of the chromatograms since a black precipitate was seen within a short period.

CONCLUSIONS

A:-

TC and OTC (analysed at pH 1.1 and 5.0 respectively) both degrade by First order. Anhydro TC (at pH 1.1) seems to follow a zero order reaction but a longer study is recommended. 4-epi-TC (at pH 3.3) also follows a first order reaction.

B:-

The tetracycline solution seems to be very unstable to light and temperature (as low as 4°C). The storage temperature should be 1-2°C. Heating at excessive temperatures results in black precipitates.

Section 4

1) Semi-preprative HPLC

2) Comparison of PRP-1 and PLRP-S columns

3) Determination of the purity of tetracyclines using PRP-1 column with solvent system 5

SEMI-PREPRATIVE HPLC

The purity of a sample of β-apo OTC, prepared in the laboratory (page 39), was examined by HPLC. Peak A (Fig.32 can be identified as OTC, and peak B was considered to be β-apo OTC. Since the two peaks were well resolved, it provided a useful oppertunity to use the PLRP-S 25cm column to isolate peak B and to identify the drug.

Since, the original buffer used in the analytical conditions was complex, which might cause problems during extraction, it was decided to employ phosphate buffer because it would not show up in a ^{13}C NMR spectrum.

The mobile phase consisted of:-

The phosphate buffer (0.07 M) at pH 5.0

Tetrahydrofuran 7.1 %

Propan-2-ol 11 %

The crude β-apo OTC was weighed and dissolved in the mobile phase. Unfortunately, its solubility was poor and the solid gradually came out of solution. Therefore it was not possible to accurately measure the percentage recovery rate. Peak B was collected over a two day period. The solution, containing peak B, was evaporated to dryness, and the solid was refluxed with hot ethanol for 30 minutes. The mixture was filtered, and ethanolic-HCl added to the filtrate until the pH reached 1.0. The solution was concentrated and allowed

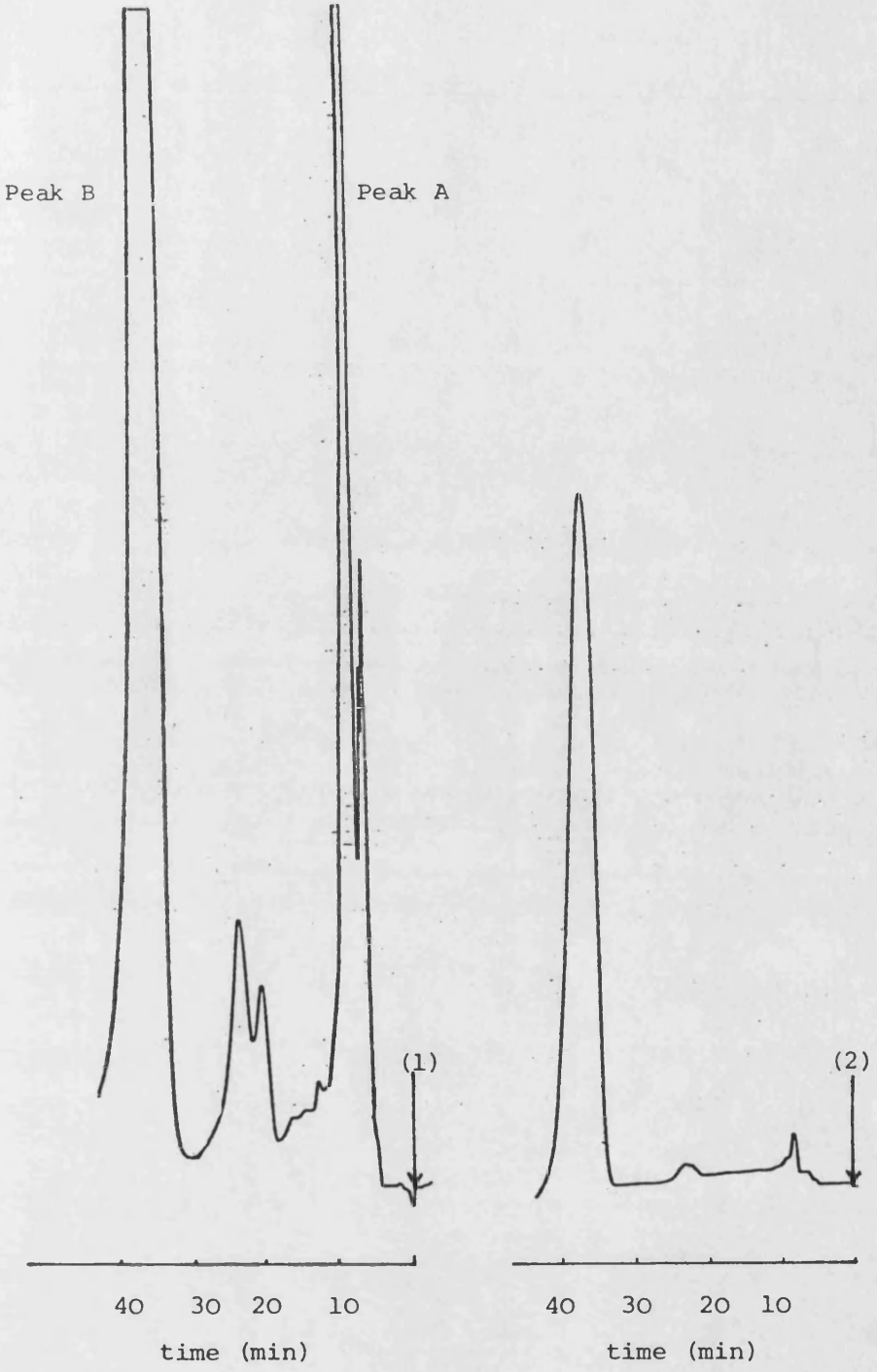


Fig. 32. Chromatograms of crude β-apo OTC (1) and the purified fraction (2). column PLRP-S 25 cm, 5ml/min, 272 nm, phosphate buffer at pH 5.0, 5mm/min, water bath at 50°C, THF 7.1 %, propan-2-ol 11 % (approximate concentrations. (1) 3.7 mg/5ml , (2) 0.5mg/500 μl)

A ^{13}C NMR spectrum was carried out on the purified sample, and the chemical shifts corresponded very well with the suggested structure. The assignments have already been discussed on page 185.

COMPARISON OF PRP-1 AND PLRP-S MATERIALS

Excellent resolution and peak symmetry values were achieved with the PRP-1 packing material for a number of tetracyclines. Unfortunately, TC and OTC eluted simultaneously immediately after the solvent front. The pH profile study failed to produce any significant resolution changes between the two drugs.

The polymer PLRP-S shares most of the physical properties of PRP-1 except that the average particle size is 8 um rather than 10 um. Reduction in particle size always improves column effeciency because it is a major factor in the terms that contribute to H.E.T.P. (height equivalent to a theoratical plate), which determines peak width. Some improvement in the resolution between OTC and TC could therefore be expected. The optimum mobile phase had to be modified for better resolution. The back pressure with the PLRP-S column was of the order 200 psi. This caused a base line fluctuation at 0.8 AUFS, which was overcome by connecting the PRP-1 column in series.

The chromatographic results are listed in Table 47 and the chromatograms are shown in Fig. 33.

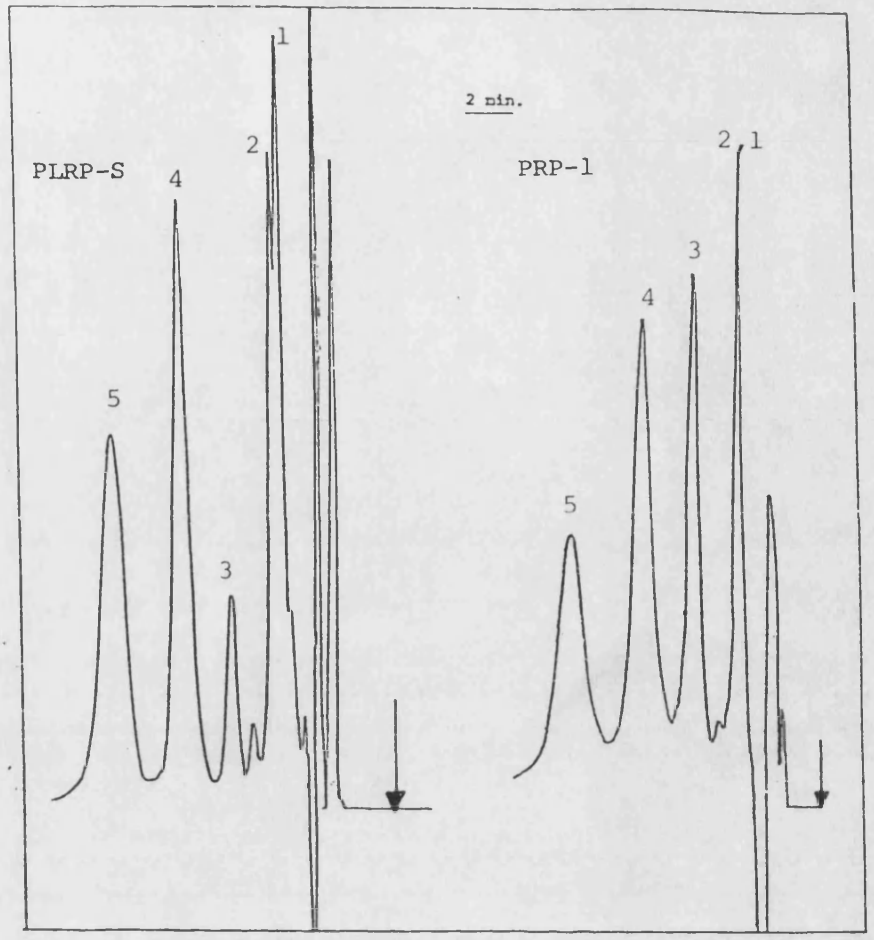


Fig. 33 The chromatographic resolution of tetracyclines on two different types of columns PRP-1 and PLRP-S. The mobile phase as described in the experimental section.

1 oxytetracycline (OTC)
2 tetracycline (TC)
3 demethyl chlortetracycline
4 methacycline
5 minocycline

Table 47 Chromatographic results obtained using two different makes of polymeric columns (PRP-1 and PLRP-S)

	Kappa		No. of plates		Total time	
	PRP-1	PLRP-S	PRP-1	PLRP-S	PRP-1	PLRP-S
OTC	1.57	1.1			6 min	6.5-7 min
TC		1.22				
DMCTC	2.92	1.96	670.4	1024.7		
METHA	4.43	2.87	653.1	1218.9		
MINOCIN	6.57	4.10	432.3	758.4		

DMCTC = demethyl CTC; METHA = methacycline; MINOCIN = minocycline

CONCLUSIONS

Improved resolution between OTC and TC was achieved with the PLRP-S packing material, but the two peaks were less than 50% resolved. Plate count was improved while the total chromatographic time remained fairly constant. It is possible that resolution could be improved further by weakening the mobile phase to increase the retention of OTC and TC, to increase the distance between the two peaks.

DETERMINATION OF THE PURITY OF TETRACYCLINES USING PRP-1 COLUMN WITH SOLVENT SYSTEM 5

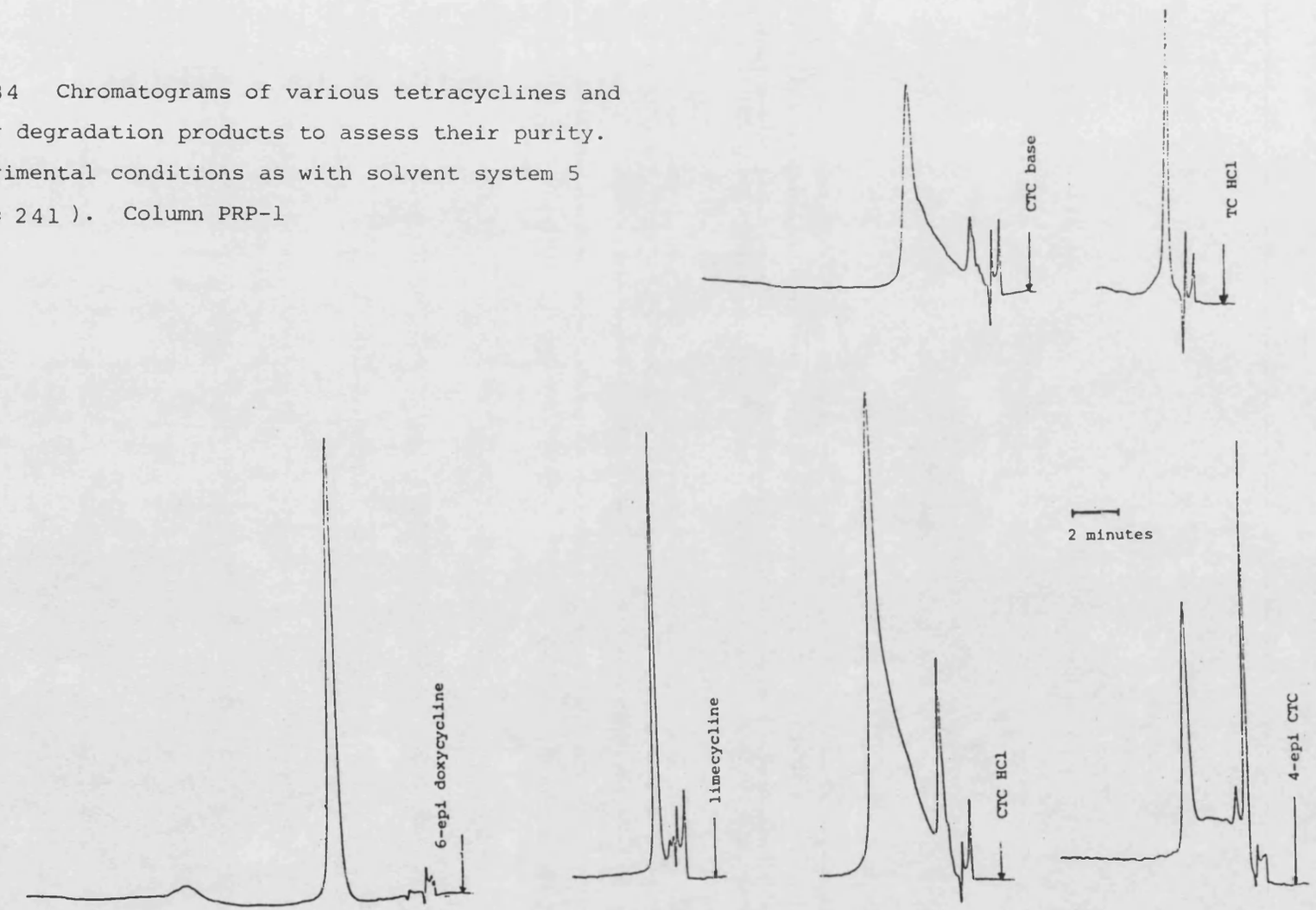
It was decided to run various tetracyclines on the PRP-1 column with the solvent system 5 to check the purity of the drug samples. TC, OTC, 6-demethyl CTC (DMCTC), methacycline (METHA) and minocycline (MINOCIN) have already been analysed.

As described earlier, TC and 4-epi TC were only resolved after the strength of the organic modifierin the mobile phase was reduced to 25%. The tetracycline sample showed a minor 4-epi TC peak. No anhyro TC peak was visible. The anhydro TC sample displayed one major peak and a peak of low intensity, the latter signal being due to the parent compound (TC HCl).

Oxytetracycline, methacycline and minocycline displayed one major peak as did doxycycline and lymecycline. 6-epi doxycycline sample displayed a low intensity peak (Fig. 34) near the position of the parent compound (doxycycline).

Chlortetracycline sample (both hydrochloride and the base) seemed impure (Fig. 34). The impurities constituted a broad band which formed theascending part of the main peak.

Fig.34 Chromatograms of various tetracyclines and their degradation products to assess their purity. Experimental conditions as with solvent system 5 (page 241). Column PRP-1



In order to increase the number of plates available for resolution, two PRP-1 columns were connected in series. The connection was made with a micropore stainless steel tube of shortest possible length. Both of the columns were kept in the water bath at 42°C. The mobile phase was formulated as follows:-

20 ml solution A (page 233) and 28 ml of solution B (page 233) were mixed together and made up to 100 ml using double distilled deionised water. The pH of the mobile phase prior to the addition of the organic modifiers was 5.7. The following proportions of the organic solvents were added to the mobile phase:

tetrahydrofuran = 9 %

propan-2-ol = 17 %

The flow rate was 1.1 ml/min, chart speed 10 mm/min and the absorbance was recorded at 272 nm.

A tetracycline sample, prepared few days previously, was injected on to the column (see Fig. 35). With the help of standards, it was possible to identify TC and epi TC, iso TC, anhydro TC and epi anhydro TC degradation products.

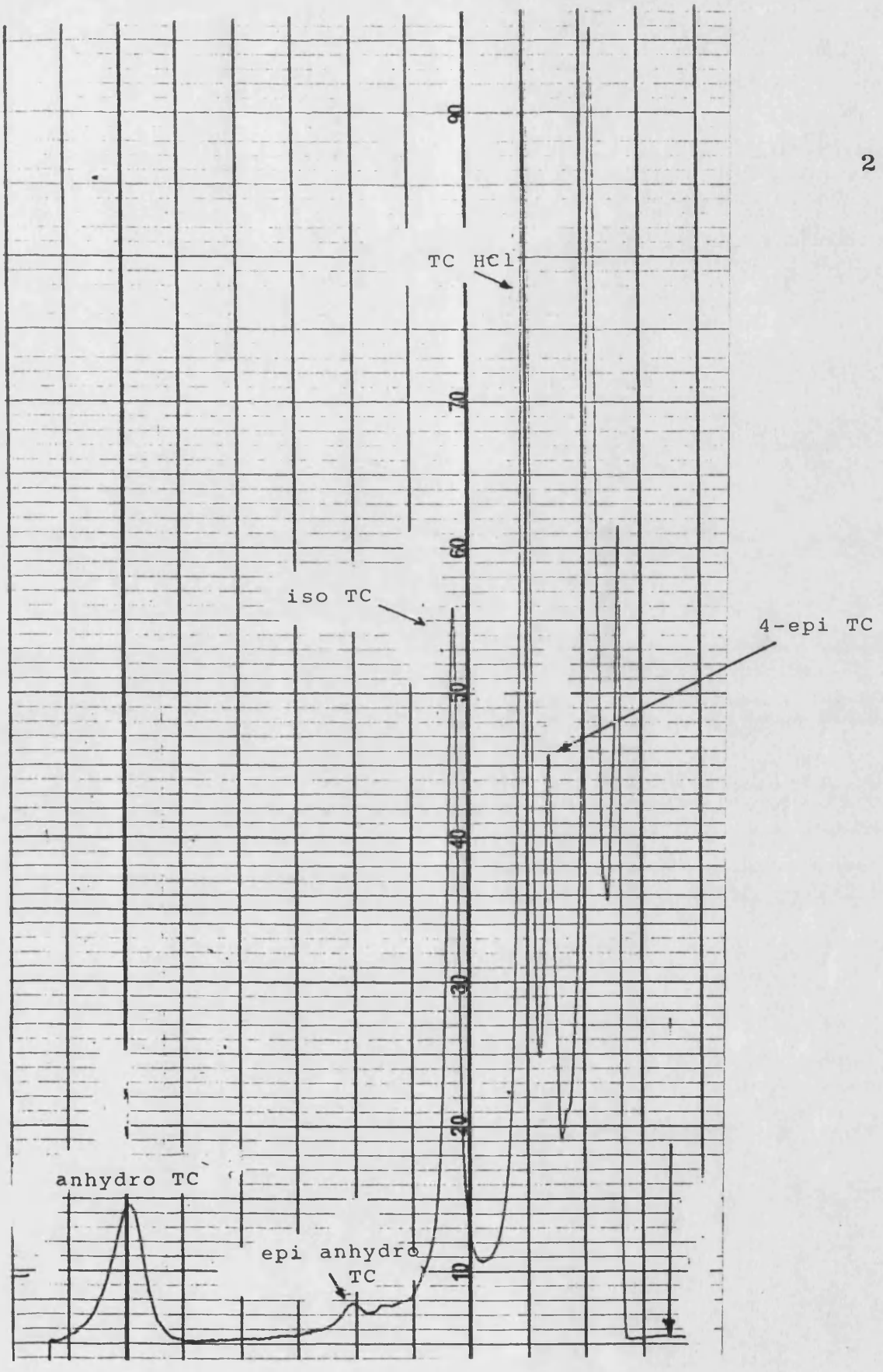


Fig. 35 Chromatogram of a tetracycline HCl sample using two PRP-1 columns (15cm) joined in series. chart speed 10mm/min

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